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, phytohormones 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[1] and differentiation[2]. The role of amino acids in growth and differentiation is known to a considerable extent[3,4]. 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[5]. Figure 1 depicts how different amino acids, supplied exogenously, affect proliferation and differentiation in *Brassica* culture[6]. 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*[7] and *Cicer arietinum*[8]. 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[8-10].

Polyamines play an important role in cell division and differentiation in eukaryotes[11,12]. Rapid accumulation of polyamines occurs concurrently with the initiation of cell division and the inhibition of polyamine biosynthesis induces differentiation[13-15]. Polyamines are implicated in

*For correspondence. (e-mail: sipra@jnuniv.ernet.in)

a variety of physiological processes like flower development, plant defence and somatic embryogenesis[16]. Some authors have even postulated polyamines as a type of plant growth regulator or hormonal second messenger[17]. 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[18,19]. 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[20].

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

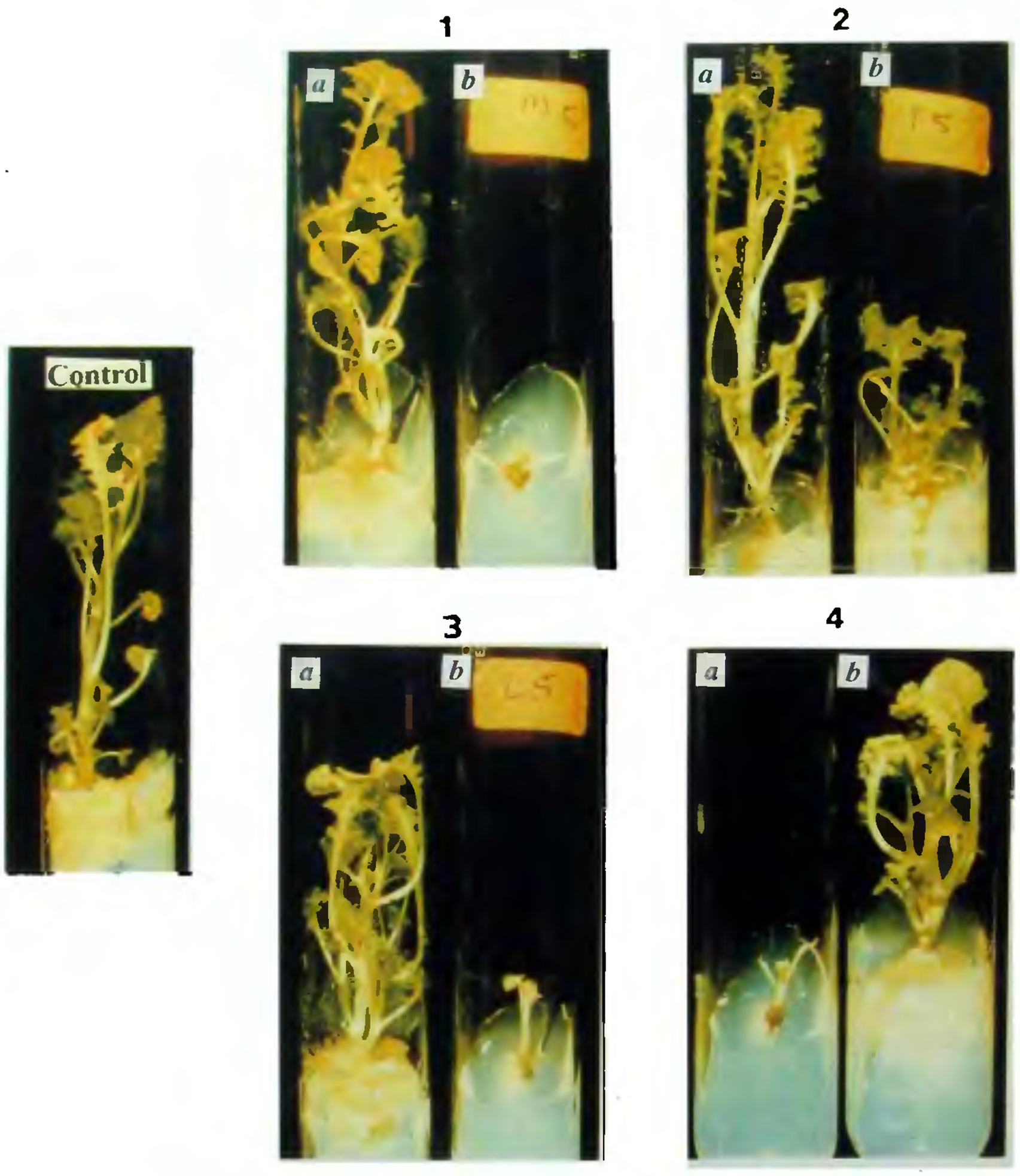

Figure 1. Effect of amino acids on *in vitro* growth of shoot apices of *Brassica juncea*. The excised shoot tips were placed on MS medium supplemented with 6-benzyl adenine (8.8 μM) and 1-naphthaleneacetic acid (2.68 μM). In addition, various amino acids, viz. methionine (1), threonine (2), lysine (3) and isoleucine (4) were added to the control medium at lower concentration, 0.5 ppm (*a*) and at higher concentration, 5 ppm (*b*).

known to influence cell commitment and cell determination[21]. Several auxin-regulated genes have been characterized and their possible roles in different cellular processes have been determined[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[23,24]. Importance of ethylene in *in vitro* cultures has been widely reported *vis-à-vis* growth and differentiation[25,26].

Ethylene influences growth and differentiation of *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 *Zea mays*[27], *Brassica campestris*[28] and *Brassica juncea*[29]. Figure 3 shows the effect of $AgNO_3$ on *in vitro* regeneration of *Brassica juncea*[6].

Cell division marker enzymes

Enzyme glyoxalase 1 which catalyses the transformation of methylglyoxal and glutathione to S-lactoylglutathione is converted to D-lactic acid by glyoxalase II[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[31]. The glyoxalase I has been correlated to cell division in *Datura*[32], coconut[33], soybean[34] and *Brassica*[35]. Glyoxalase I level was reported to be high in proliferating cells of *Brassica* and declined during differentiation induced by inhibitors of polyamine and ethylene biosynthesis[35].

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[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.

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[37]. Likewise, Stirn and Jacobsen[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[39].

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.

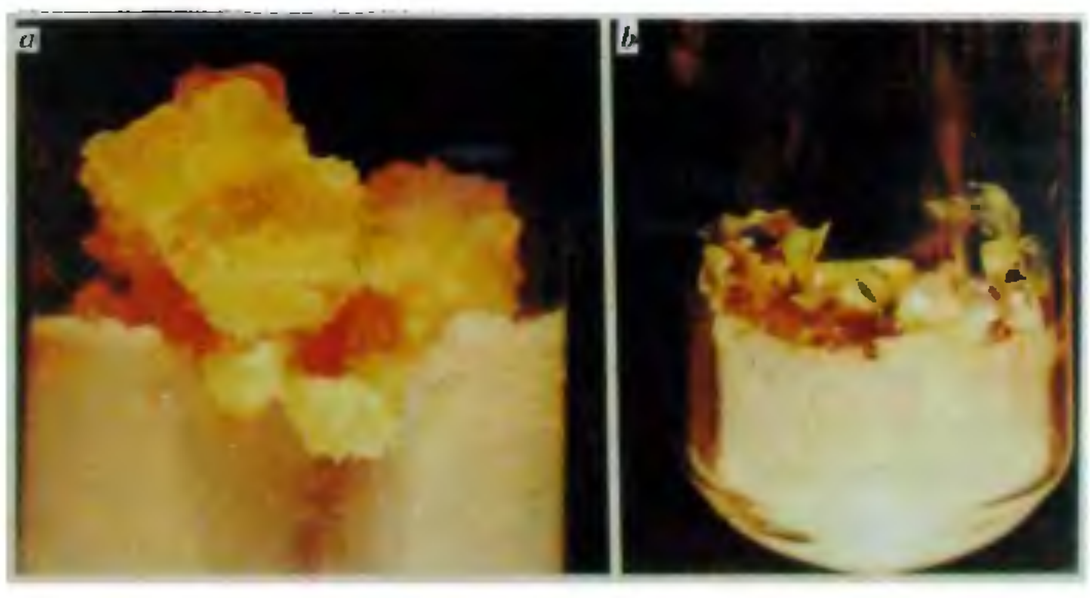

Figure 2. Growth and differentiation of *Brassica* culture on spermidine (Spd) containing medium. *a*, Basal medium with hormones (NAA, 5.37 µM; BA, 1.4 µM) and Spd, 1 µM; *b*, Basal medium with Spd (1 µM).

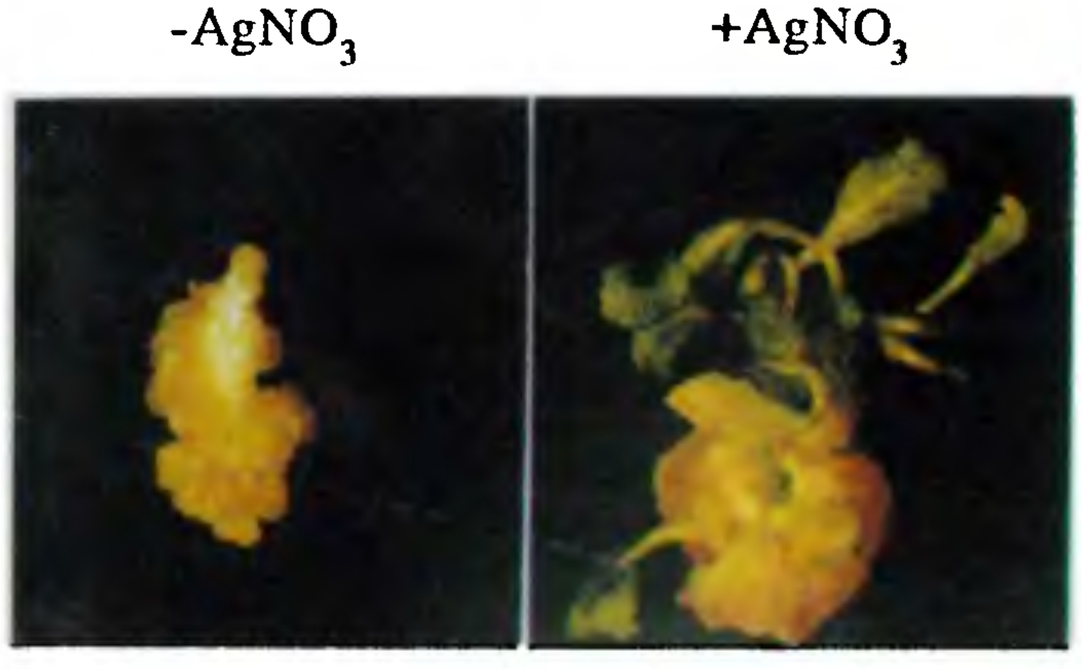

Figure 3. Effect of $AgNO_3$ on shoot differentiation. The hypocotyl explant was placed on MS medium with or without $AgNO_3$ (50 µM).

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[40]. Yoshida *et al.*[41] 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 cDNA and aldose reductase cDNA is limited to embryogenic culture of barley[39]. Two cDNA fragments, G35 and G36, have been isolated from tomato by mRNA differential display and it has been shown that they are modulated in time- and growth regulator-dependent manner during early phase of *in vitro* shoot determination[42].

Plant cell wall proteins

Plant cell wall plays an important role in developmental processes. It is considered that information of fate determinants resides on the cell wall. Extensive studies have been done on proteins in plant cell wall[43]. There are five major classes of cell wall proteins: hydroxyproline-rich glycoproteins (HRGPs), proline-rich proteins (PRPs), glycine-rich proteins (GRPs), arabinogalactan proteins (AGPs) and solanaceous lectins. The specific 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[44]. 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[45]. 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[46].

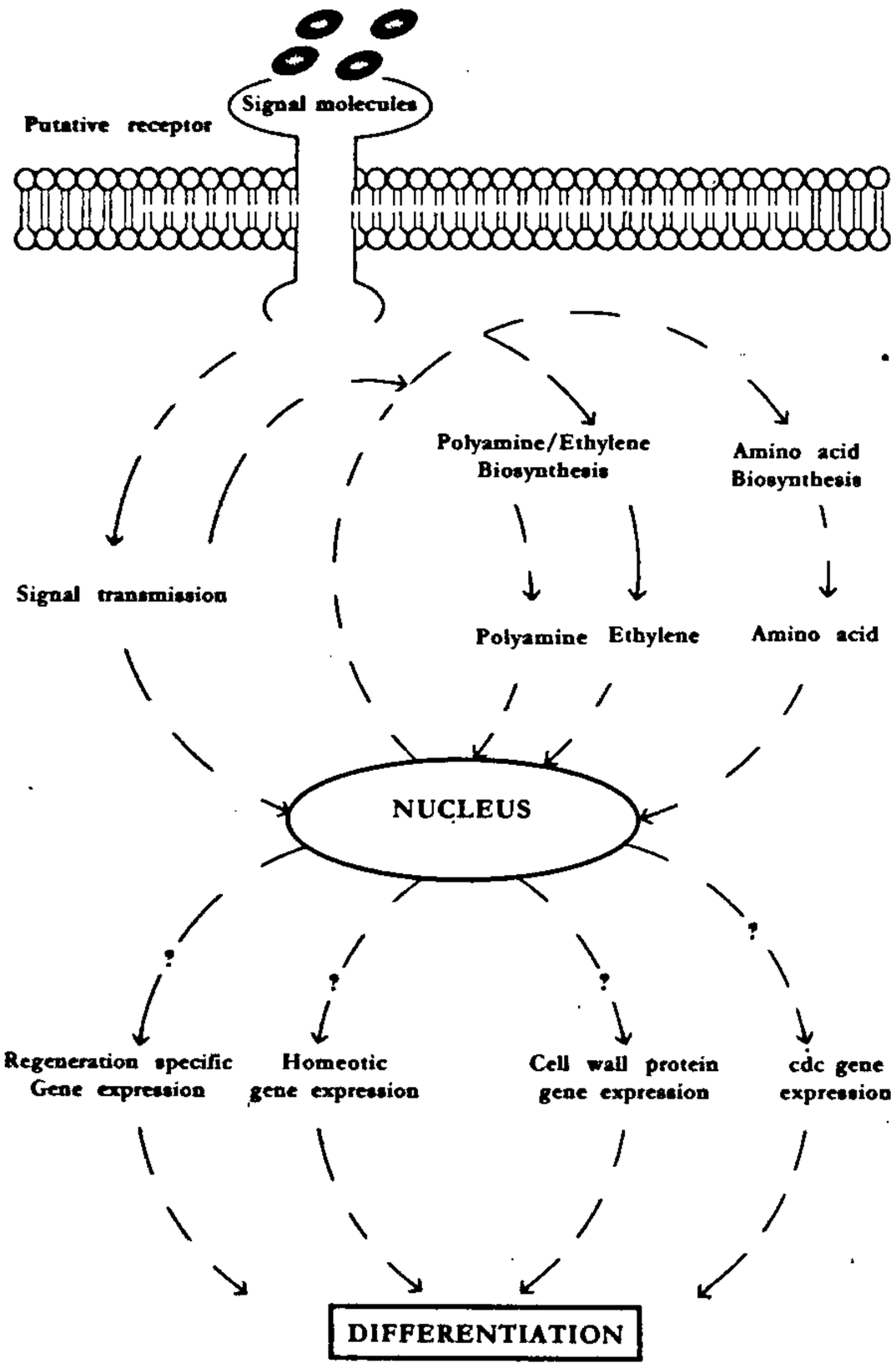

Figure 4. Schematic diagram representing the current understanding of differentiation process in plant tissues.

Cell-cycle and cell-cycle division genes

Cell proliferation and differentiation are mutually exclusive phenomena. The start point of cell cycle, G1 phase, decides whether the cell will divide or differentiate. Differentiation may be initiated with proper signals both at G1 and G2 phases of the cell cycle[47]. For example, critical events for the induction of tracheary differentiation in *Zinnia elegans* parenchyma occurred during early G1 phase[48]. Supplementation of trigonelline, which causes arrest in G1/G2[49] and theophylline, which is reported to induce a block of cell cycle in G1 in roots of *Haplopappus*[50] induce differentiation in *Brassica*[51]. Network of genes and their products play a crucial role in cell division and differentiation. For instance, a nucleolar protein fibrillanin increases with increased nucleolar activity in G2 and probably decreases when nucleolar activity declines during differentiation[52]. Another report shows a nucleolin-like protein NucMs1 is tightly linked with cell proliferation but has no trace in the cell differentiation process[53]. Many cell division cycle (*cdc*) genes, have been cloned and sequenced in animal system[54]. In plants, *cdc* homologues have also been isolated and sequenced[55]. In animals, cdc2 level could be stringently correlated with the proliferative state of cell[56,57]. In higher plants, there is a positive correlation between cdc2 level and meristemic activity, though cdc2 mRNA is also found in non-dividing tissue[58]. *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[59].

In dividing tobacco protoplasts, the proliferation marker enzyme-glyoxalase I[35] was induced in a phase-dependent manner prior to the G2/M phases of the cell cycle. The

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[60]. 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*[61]. Several homeotic genes have also been isolated from maize[62], rice[63], *Arabidopsis*[64] and soybean[65]. The ectopic expression of the homeobox genes caused abnormal leaf development in transgenic plants[66]. The plant homeotic genes are also directly involved in embryo development. A rice homeobox 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[67]. 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[68]. Maize homeobox gene knotted-1 (*kn-1*) is a useful marker of meristem activity. The expression of *kn-1* is reported on the dome of all meristems[69]. The first detectable expression of *kn-1* occurs during embryogenesis before shoot meristem organization[70].

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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