1. GENERAL INTRODUCTION

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GENERAL INTRODUCTION

Biotechnology has come to a stage where by complementing some of the age-old practices of breeding it can produce novel and improved plants and animals that can serve better to the human being's requirements. The techniques of cellular and subcellular engineering such as gene splicing and recombinant DNA and its cloning, hybridomas and monoclonal antibodies, protein engineering, industrial fermentation, plant tissue culture and nitrogen fixation, phytomass production for biofuels etc have advanced very greatly. This has been possible due to the availability of better equipments and the advances in our basic knowledge of various biological processes.

1.1. Biotechnology and tissue culture:

Plant biotechnology, however is racing ahead in geometric proportion because unlike the biotechnology of animals, plant biotechnology raises considerably fewer social, moral and legal issues. Tissue culture is one of the important component of plant biotechnology because of following developments -

1) Micropropagation for biomass, energy production and elite plants of high quality and productivity (Boulay, 1987).
2) Production of disease-free plants by shoot tip culture (Westcott, 1983).

3) Selection of mutants resistant to pests, pathogens, adverse soil conditions, drought, temperature, herbicides etc at cellular level (Chaleff, 1981; Hanson, 1984; Maliga, 1984; Dix, 1985; Wenzel et al., 1987).
4) Production of haploids through anther/pollen culture for rapid production of 'pure' lines (Chu, 1982; Maheshwari et al., 1982; Dunwell, 1986; Lyne et al., 1986; Keller et al., 1987).
5) Wide hybridisation through intra or interspecific protoplast fusion (hybrid or cybrid) and embryo rescue (Cocking, 1983a and b; Fassuliotis and Nelson, 1986; Mallikarjuna et al., 1986).
6) Transformation of plants through either genetically engineered Ti plasmid of Agrobacterium tumefaciens, electroporation or microinjection (Schroder et al., 1985; Horsch et al., 1987; Lorz et al., 1987; Potrykus et al., 1987).
7) Conservation of germplasm through cryopreservation of shoot meristem or other suitable explants (James, 1983; Withers, 1985, 1986; Kartha, 1987).

Conventional plant breeding has no doubt, made considerable progress in the improvement of yield and the quality of traits of a wide range of crop species leading to the green revolution almost throughout the world. However, about 50% of this increase in yield is mainly due to the use of selected superior varieties and the rest due to better agronomic practices based on the scientific knowledge.(Bingham, 1981). In crops like cotton,

rice etc., however, plateu has reached and unless some novel approaches are made, chances for further improvement appear very remote (Bright et al., 1983).

The in vitro methods for manipulating plant cells, have progressed to a state where a considerable contribution towards crop improvement, can still be made for those species for which conventional plant breeding has been less successful (Bright et al., 1983). Thus the advent of in vitro plant cell culture has provided us with a powerful technology for possibilities of extrapolating methodology and knowledge from microbial and mammalian cell genetics to plant systems. The possibility of obtaining plants from single cells under controlled culture parameters has changed our way of approach about plant genetics and crop breeding (Negrutiu et al., 1984) as entirely new and very useful concepts are being born and employed.

During the last few years, two major achievements have been accomplished towards the successful introduction of foreign genetic information into plants (Negrutiu et al., 1984). One is the successful isolation and culture of plant protoplasts and second is transfer of cloned genes (Ohyama, 1983; Gamborg et al., 1981; Schreier et al., 1985, Fraley et al., 1986) through vectors like Ti plasmid of the soil pathogen Agrobacterium tumefaciens (Rogers et al., 1985). The foreign genes, when tagged with some selectable marker genes like resistance to antibiotics, selection and monitoring of

transformed plant material can be achieved with ease (Wilke-Douglas et al., 1986; Schreier et al., 1985).

Recently, progress in engineering glyphosate (herbicide) tolerance into plants has been achieved by over expression in Petunia hybrida of a chimeric gene (Shah et al., 1986). This gene from Escherichia coli, tagged to cDNA encoding the transit peptide sequence, has been shown to be imported into chloroplasts to give a resistant enzyme: 5-Enolpyruvylshikimate-3-phosphate synthase (della-Cioppa et al., 1986 and 1987). Comai and his co-workers have also engineered glyphosate tolerance into tobacco through expression of bacterial gene (Comai et al., 1985). Similarly, there are reports of successful transfer of gene for luciferase in tobacco plants (Ow et al., 1986), introduction of phaseolin gene into tobacco (Sengupta-Gopalan et al., 1985), expression of chimeric gene containing neomycin phosphotransferase in sunflower (Everett et al., 1987) and foreign gene into Douglas-fir (Dandekar et al., 1987). These results of genetic engineering research have now generated demands of (a) isolation of useful genes of agronomic importance (like nutritional enhancement, photosynthetic efficiency, tolerance to adverse conditions etc) whose introduction into crop plants would increase their agronomic performance (Perani et al., 1986); (b) development of regenerating systems comparable to the model systems like tobacco, carrot, petunia etc (Yeoman, 1986). Work presented in this thesis relates to the basic studies of the second aspect.

1.2. Plant development and gene expression:

The plant development involves both growth and differentiation. The term growth is assigned to quantitative changes occurring during the course of development and can be defined as an irreversible change in the size of cell, organ or a whole plant. Nevertheless, during development, there appears not only the quantitative differences in the number, size and arrangement of cells within different organs, but also the qualitative differences between cells, tissues and organs which is termed as differentiation (Wareing and Philips, 1982). On the other hand, according to Burgess (1985), the plant development encompasses three types of sequential processes. First, formation of new cells by cell divisions, followed by a growth or cell enlargement. Finally, the cells differentiate into their mature and respective specialized states. Broadly, the term differentiation, can be applied to any situation in which the meristematic cells give rise to two or more types of cell, tissue or organ which are qualitatively different from each other.

If this is the case, then is it possible to study the way in which plant cell differentiate and develops into functional congregate ? The answer is yes and the studies can be made at different levels.

It is axiometic, for instance, that the cell development solely depends upon the regulated expression of genetic material (Matters and Scandalios, 1986). There are considerable evidences

to advocate that highly differentiated cell types within a plant body retain all the genetic information required to specify the structure and function of an entire plant. This is called totipotency, a behaviour first observed by Steward et al., (1958). This was subsequently noted by various workers, as complete plant regeneration has been successfully achieved using explants of roots (Winton 1968); cotyledon (Hu and Sussex, 1971); hypocotyl (Kamat and Rao, 1978); stem (Hill 1967); petiole (Clare and Collins, 1974; leaf (Pareek and Chandra 1978); shoot apex (Kartha et al., 1976); inflorescence (Hussey, 1975); flower petals (Heuser and Apps, 1976); anther (Guha and Maheshwari, 1964); ovules tissue (Kochba and Spiegel-Roy, 1973); embryos (Nag and Johri, 1969) and nucellus (Rangaswami, 1959). This fact implies that within any given cell of the plant body, only part of the genetic information is being expressed at any time, depending upon its position and function. This indicates that the development involves the activity of specific groups of genes which in turn controls the synthesis of particular enzymes and other proteins characteristic of specialized cells. However, gene expression in plants is known to be altered by hormones, even at transcriptional levels (Burrell et al., 1986) e.g. auxin induced very rapid induction of mRNA even prior to initiation of cell elongation (Theologis, 1986).

Differentiation can also be expressed in terms of biochemical activities. Specific biochemical specialization is limited to

quite small populations of cells. Therefore, it is reasonable to assume that the structural as well biochemical changes observed during the ontogeny of plant organs must be preceeded by or concomitant with changes in the activities of various enzymes (Nave and Sawhney, 1986). Several studies have documented a correlation of the occurrance of certain enzymes and their multiple forms - isozymes during the development and differentiation of tissues and organs (Scandalios, 1983).

1.3. Recalcitrant plant regeneration - a basic problem:

Plant regeneration has been found to be quite difficult in many of the agriculturally important crops (Bhojwani and Razdan 1983; Wilke-Douglas et al., 1986), specially in cereals (Cocking, 1985; Maddock, 1985; Vasil, 1985; Ozias-Akins and Lorz, 1984, Lorz et al., 1987; Wernicke and Milkovits, 1987), certain legumes (Flick, 1983); and important mature hardwoods (Bonga, 1982; Bhojwani and Razdan, 1983). Numerous reports of complete plant regeneration observed in good number of plant species belonging to various families demonstrate that this is not a phenomenon restricted to just a few taxa (Yeoman, 1986). However, many of our crop plant of agronomical as well as horticultural interest are still recalcitrant due to unknown reasons. It is now well documented that genetic factors also contribute to the response of plant tissue in culture (Lee, 1984; Dunwell, 1981; Raquin, 1982). Further even

Though all cells within an organism are considered to be of the same genotype, there are striking differences from cell to cell and from organ to organ within a plant in its ability to regenerate in cultures (Brown and Atanassov, 1985; Ammirato, 1986). In cotton, for example, wide range of callus initiation, proliferation and maintenance responses found reflect the degree of genotypic diversity within the germplasm collection (Lee, 1984). Virtually nothing is known about the principles governing the crucial transition from responsiveness to apparent lack of responsiveness which occurs during cell differentiation (Wernicke and Milkovits, 1987). At present, it is not clear, how far this lack of morphogenetic response obtained with so many plants, particularly legumes, cereals and other woody trees is due to deficiencies in nutrient medium or to other physical and chemical factors connected with the method of culture. However, according to Halperin (1986), this refractory response of some cells of explants or subcultures seems to be due to a certain block in the regeneration process. The detailed molecular analysis of such blocking systems have still not been achieved due to certain eccentricities in the plant systems (Sanchez-Martinez et al., 1986), mainly due to more complexity than that of bacterial systems (Cocking, 1986). In this context, it has been suggested that genetic improvement may prove more useful than manipulation of environmental variables in the establishment and optimisation of culturing strategies (Lazar et al., 1984). Part of the failure is due to lack of fundamental understanding

on the biochemical and molecular aspects of plant growth and development (Yeoman, 1986). Ever since Skoog and Miller (1957) demonstrated that the relative ratio of auxins and cytokinins determines the kind of organogenesis (Shoot or root) in tobacco pith callus, very few basic studies have attempted the question why certain plant system responds to this empirical approach of auxin/cytokinin ratio, while others do not (Murashige, 1974; Evans et al., 1981; Ammirato, 1986). In this context, Wernicke and Milkovits (1987) suggested correlation of the loss of responsiveness with the uncoupling of auxin from the control of cell cycle, probably through receptor sensitivity to hormones (Trewavas, 1982a; Trewavas and Cleland, 1983; Starling et al., 1986). However, probability of impaired polyamine metabolism too, cannot be ruled out as polyamines has been shown to play an important role in plant development including growth and differentiation (Cohen et al., 1979; Bagni et al., 1981; Apelbaum et al., 1982; Desai and Mehta, 1985; Smith, 1985; Vansuyt and Zinsou, 1986; Kaur Sawhney and Galston, 1987).

1.4. Lack of appropriate system:

It has been realized that the lack of progress in understanding the process of plant morphogenesis and development is due to the lack of adequate well defined experimental system (Thorpe and Biondi, 1981; Tran Thanh Van and Trinh, 1986; Yeoman, 1986). The multiplicity of correlation between organs, tissues and cells in intact plants hinders in locating precisely

the recognition sites as well as the target cells for studies on pathways leading to organ differentiation. Thus, systems less complicated than integrated intact plants have been used to evaluate fundamental studies on morphogenesis. These include organ fragments isolated cells, protoplasts and thin cell layers. Further in order to closely define the control point of morphogenesis developmental mutants analogous to the embryo-lethal mutant of Arabidopsis thaliana (Meinke and Sussex, 1979; Meinke, 1986; Fang and Mayerowitz, 1987) could be utilized. However, all these experimental systems suffer from their intrinsic disadvantages (refer Chapter 5 for further details).

1.5. Epiphyllous bud outgrowth - system to study plant development:

In this regard it seems that the epiphyllous buds of Kalanchoe mortagei offers an excellent experimental material fulfilling criteria of Thorpe (1979) to study molecular aspects of morphogenesis and plant development. K. mortagei Raymond Hamet and Perrier is a succulant garden plant belonging to the family Crassulaceae. The oppositely deccusate leaves, in their notches on either sides, contain a large number of meristemoids (40-70 per leaf) which develops into complete plantlet with some trigger. Besides these bud primordia, de novo differentiation of large number of shoot buds can also be readily achieved from the cut part of the leaf simply by pulse application of cytokinin. This system offers following advantages for basic studies pertaining to plant development:

1) Known site of bud development, that means, the development of complete plantlet occurs only at predetermined specific sites called notches.
2) Rapid responses, that means the complete development of buds occurs within about 10 days after isolation and subsequent incubation of explants.
3) Ease of experimental manipulations of the explant. The bud outgrowth does not require any exogenous nutrient medium as only water is adequate for entire experimentation.
4) Availability of many variants for the induction of bud outgrowth. Depending upon the species, the epiphyllous bud growth occurs on either intact (attached) leaf (*B.diagremontianum*, *B.tubiflorum*) or isolated one (*K.mortagei*) or both (*B.calycinum*).

1.6. Studies undertaken:

Based upon these facts, the present study was undertaken to examine following events underlying epiphyllous bud outgrowth in *K.mortagei*.

1) Anatomical studies of epiphyllous buds during their dormancy and subsequent reactivation of growth.
2) Hormonal regulation of the bud growth:
   a) correlative inhibition and bud dormancy.
   b) polarity of epiphyllous bud outgrowth.
3) Quantitative and qualitative changes of soluble proteins during the course of bud reactivation and growth.

4) Involvement of IAA oxidase including phenolic metabolism in epiphyllous bud outgrowth.

The thesis is presented in seven chapters. Brief introduction of each of the aspects with its own methods employed are described separately. The results obtained in each of the approaches are also discussed individually in light of pertinent literature in respective chapters. This is followed by a general conclusions in chapter VII.

It seems that the above mentioned basic information on the epiphyllous buds of K. mortagei will put this experimental system at the level comparable to that of the somatic embryogenesis of carrot (Sung and Okimoto, 1983) to prove detailed molecular analysis of yet unexplored developmental process in plants i.e. bud dormancy and its reactivation process.

In this thesis we present evidences for a molecular marker for epiphyllous bud meristem development as has been shown for embryogenesis (Sun et al., 1978; Evans et al., 1979), germination (Higgins et al., 1976; Weir et al., 1980), root nodule formation (Verma et al., 1974).