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Looking for isoforms of enzymes related to *in vitro* morphogenesis in *Nicotiana tabacum* L.

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Abstract

Peroxidase (PRX, E.C.1.11.1.7), Esterase (EST, E.C.3.1.1.1), Acid Phosphatase (ACP, E.C.3.1.3.2), α-Amylase (E.C.3.2.1.1) and Malate Dehydrogenase (MDH, EC 1.1.1.37) enzymes were studied through native activity gel electrophoresis in three in vitro developmental stages (non regenerating callus, regenerating callus and leaves of regenerated plantlets) of tobacco (Nicotiana tabacum L.) to look for up- or down regulation of specific isoforms by densitometry scanning to correlate with changing developmental stage. Certain isoforms of Peroxidase, Esterase and Acid Phosphatase, were found to be either up- or down regulated in the course of dedifferentiation, while two other enzymes, viz. α-Amylase and Malate Dehydrogenase showed little alteration in activity in the three developmental stages under study.

Keywords: *In vitro* developmental stages, isoenzymes, tobacco.

Introduction

Research on molecular mechanism of plant developmental biology is gaining momentum in recent times. The central problem in plant developmental biology is to understand the structural and regulatory roles of the genes interplaying in differentiation. Structural genes code for RNA molecules. The mRNAs subsequently become destined for the synthesis of the structural proteins vis-à-vis enzymes necessary for cell structure and metabolic activities. Regulatory genes, on the other hand, control the expression of structural genes at the temporal, spatial, and quantitative level. During plant development a well-defined temporal and spatial orchestration for gene expression demands interaction between regulatory and structural genes. Developmental studies with isoenzymes have provided considerable insight into the role of structural genes and into the mechanisms that control enzyme synthesis during cell differentiation, consistently supporting the concept of differential gene action during this process[1].

The usual experimental approach of plant developmental biology is to look for gain- / loss-of-function mutants[2] and / or study specifically designed transgenics[3], followed by genetic and molecular analysis[4]. However, apart from the *Arabidopsis* system, obtaining mutants at will is not possible in most of the plant systems. Plant cell and tissue culture or the *in vitro* approach, however, is a good alternative to study plant morphogenesis. This approach further gives the opportunity of elucidating two remarkable modes of regeneration of *in vitro* plant system, viz. organogenesis and somatic embryogenesis, which can occur either directly (from explant), indirectly (callus-mediated) or by both in few plant systems[5]. Since both organogenesis and embryogenesis are extremely dynamic processes and triggering of the key molecular and biochemical switch takes place much earlier in temporal scale than the actual manifestation of the phenomenon; a time – lapse approach for the plant materials under study in pre standardized media may help to look for the altered expression of isozymic profile(s) associated with *in vitro* plant morphogenesis.

The present study aims to study certain development specific isoenzymes like Peroxidase, Esterase, Acid Phosphatase, α-Amylase and Malate Dehydrogenase through native activity gel electrophoresis in three *in vitro* developmental stages (callus, regenerating callus and leaves of regenerated plantlets) of tobacco (*Nicotiana tabacum* L.), the model tissue culture material. The objective being to identify specific isoforms of the aforesaid enzymes following their up- or down regulation as revealed by densitometry scanning in correlation with altered developmental stage.

Material and Methods

Plant material: An *in vitro* maintained tobacco (*Nicotiana tabacum* L. var. Jayasri) plant system was used in present study. Three contrasting developmental stages were selected: i. callus (freshly initiated from cut surface of leaves of *in vitro* grown plants), ii. regenerating callus (at the onset of green shoot bud-like structure initiation); and iii. leaves of regenerated plantlets (figure-1). Callus was initiated and maintained in dark in MS (Murashige and Skoog) basal media[6] supplemented with NAA (1-Naphthaleneacetic acid, 1.0 mg/l) and Kn (Kinetin (0.5 mg /l). One set of proliferating callus was sub cultured in MS basal media supplemented with NAA (0.5 mg /l), BAP (6-benzylaminopurine, 0.25 mg/l) and Kn (0.25 mg /l); and cultures were put under light (2000-2500 lux, photoperiod of 16 h/day) for regeneration. Repetitive regeneration with emergence of shoot buds was obtained within three sub cultures in same

cultural condition. Similar cycle of transferring callus from proliferation to regeneration medium was continued and from those cultures emerging shoots were subsequently transferred to MS medium supplemented with Kinetin (0.5 mg/l) and IBA (Indole-3-butyric acid, 1.0 mg/l), wherein the tobacco plantlets are maintained. The pH of media was adjusted to 5.6 after addition of growth regulators but before adding the gelling agent (0.75% w/v agar); media were subsequently autoclaved at 121ºC, 104 kPa for 15 min. Tissues at their exponential growth phase (14-15 days after fresh sub culture) were harvested for protein extraction prior isoenzyme analysis.

Protein extraction and quantification: Extraction for crude protein containing the enzymes was done using 1 g of fresh samples in each case (callus, regenerating callus and leaves of regenerated plantlets) following the protocol of Wetter and Dyck[7]. Samples were homogenized at 4°C with 1.5 cm^3 of Tris-HCl buffer (0.2 M, pH 8.5) containing sucrose (1 M) and 2-mercaptoethanol (0.056 M). After centrifugation (16000 g for 20 min at 4°C) the supernatant was collected and considered as source of enzyme. Protein content was estimated by the Folin-phenol method[8]. For normalization prior performing isozymic native poly acryl amide gels, equimolar amounts of protein (100 µg) of three samples were subjected to sodium dodecyl sulphate poly acryl amide gel electrophoresis (SDS-PAGE) following Laemmli's protocol[9] with subsequent coomassie staining.

Isozymic analysis: Isozymic analyses of five enzymes – Peroxidase, Esterase, Acid Phosphatase), α–Amylase and Malate Dehydrogenase were performed after native anionic PAGE (10 %) followed by respective specific enzyme substrate activity in-gel staining following standard protocols[7,10-12]. For Peroxidase activity staining 50 µg of samples was loaded into gel while for the four other enzymes 100 µg of samples were used for each sample. Electrophoretic runs were made for 3–4 h at 2 mA per lane at 4ºC.

Analysis and relative quantification of enzyme isoforms: Analysis was done using BIO-RAD Gel Documentation System (Gel Doc 1000, version 1.5). From the scans of three different lanes (samples) of each isozymic gel peaks (bands / isoforms of respective isoenzyme) were identified on the basis of Rmf (Relative mobility factor) values followed by quantification on the basis of relative percent intensity (absorbance).

Results and Discussion

Since the present study targets towards analysis of development stage specific isoforms of different enzymes through native gel electrophoresis vis-à-vis study of up– or down regulation of the same, normalization of quantification of the absorbance of three different samples was of utmost importance. This was done initially by loading equimolar concentration of protein of all the samples followed by visualization of the polypeptides through SDS-PAGE followed by validation of relative percent intensity (absorbance) of each and every coomassie stained bands (peaks). Almost all the bands of three developmental stages under study revealed comparable percent intensity except the 55 kDa subunit of Rubisco, conspicuous for green leaves (figure-1). This approach, plausibly, paved the way for relative estimation of different enzymes, the isoforms of which could expectedly up- or down regulate controlling the events of morphogenesis.

Of all the isozymes, Peroxidase is the most intensely studied one. Growth regulator induced promotion or suppression of *in vitro* plant growth has direct consequences on isoforms of Peroxidase[13]. In the present study also Peroxidase profile altered from callus (represented by iso Peroxidase 1, 2 and 4) to leaves (represented by iso Peroxidase 1,2,3,4 and 5) via the intermediate regenerating callus (represented by isoPeroxidase 2,3,4,5,6 and 7) developmental stage (Figure-2). Of all the isoperoxidases, the slowest migratory one (band P1, Rmf 0.10), which was remarkable in intensity in non- regenerating and regenerating callus stage became almost obscure in leaves. On the other hand, bands P4 and P5 (Rmf 0.42 and 0.46 respectively) intensified many fold in regenerating callus and leaves in comparison to the non-regenerating callus. Apart from that, two fast moving isoperoxidases (bands P6 and P7) appeared only in the leaf developmental stage (figure-2). Developmental stage specific alterations in isozymic profile of Peroxidase are also observed in other *in vitro* plant systems[14-15].

Apart from Peroxidase, the biochemical alteration during *in vitro* organogenesis has been studied in a number of plant systems through analysis of isoforms of Peroxidase, Esterase, Acid Phosphatase etc[16, 17]. The isoforms of Esterase profile in the present study also showed differential response, of which gradual intensification of two bands (E3 and E4 having Rmf 0.54 and 0.56 respectively) was conspicuous from non regenerating callus to leaves via regenerating callus stage (figure-3).

Acid Phosphatase, a class of which though sometimes considered as 'non specific enzymes' yet playing important role in production, transport and recycling of inorganic phosphate, while the other class show specialized functions, particularly in shoot formation from unorganized meristematic mass of tissues[18]. The present experimental scope though does not permit to predict much but the appearance and intensification of the slowest migratory isoform of Acid Phosphatase (AP1, Rmf 0.14) (figure-4), particularly in the regenerating callus warrants further investigation as circumstantial evidence of onset of shoot bud development was evident in this stage only.

Starch-degrading enzyme α–Amylase, which otherwise have important function in mobilizing resources during embryo development[1]; and Malate Dehydrogenase, a rather ubiquitous enzyme having role in conversion of oxaloacetate from Malate and a product of housekeeping genes as well [19], both showed low number of isoforms (only two and one in case of α–Amylase and Malate Dehydrogenase respectively) and little alteration in activity in the three developmental stages under study (figures-5,6).

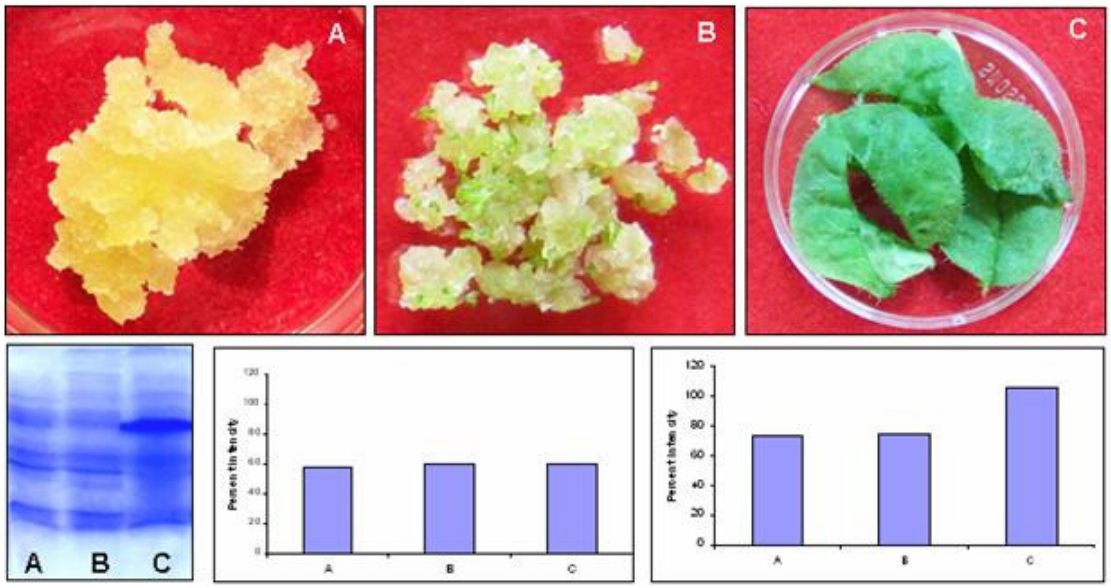

Figure-1

Three *in vitro* developmental stages of tobacco under study (non regenerating callus - A, regenerating callus – B, leaves of regenerated plantlets – C); SDS-PAGE profile of total protein/crude enzyme extract of three developmental stages with densitometry scan based percent intensity of the polypeptides, of which one representative shown in the left block showing comparable quantification in all the samples, while the right block showing gradual intensification of Rubisco (arrow marked) in the leaf sample

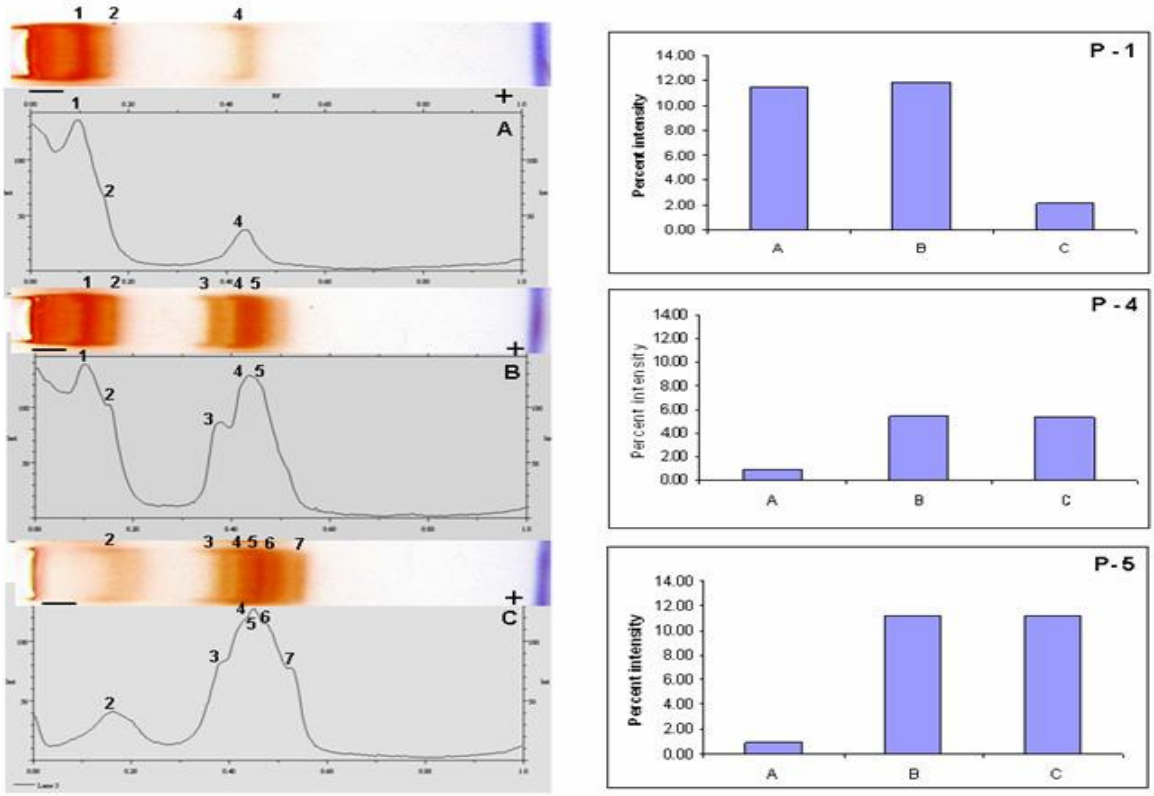

Figure-2

Isoforms of Peroxidase in three developmental stages (non regenerating callus - A, regenerating callus – B, leaves of regenerated plantlets – C) and their respective densitometry scanning; respective band / peak is numbered of which three significant isoperoxidases (P-1, P-4 and P-5) showing relative up- down regulation (based on percent intensity) in different developmental stages

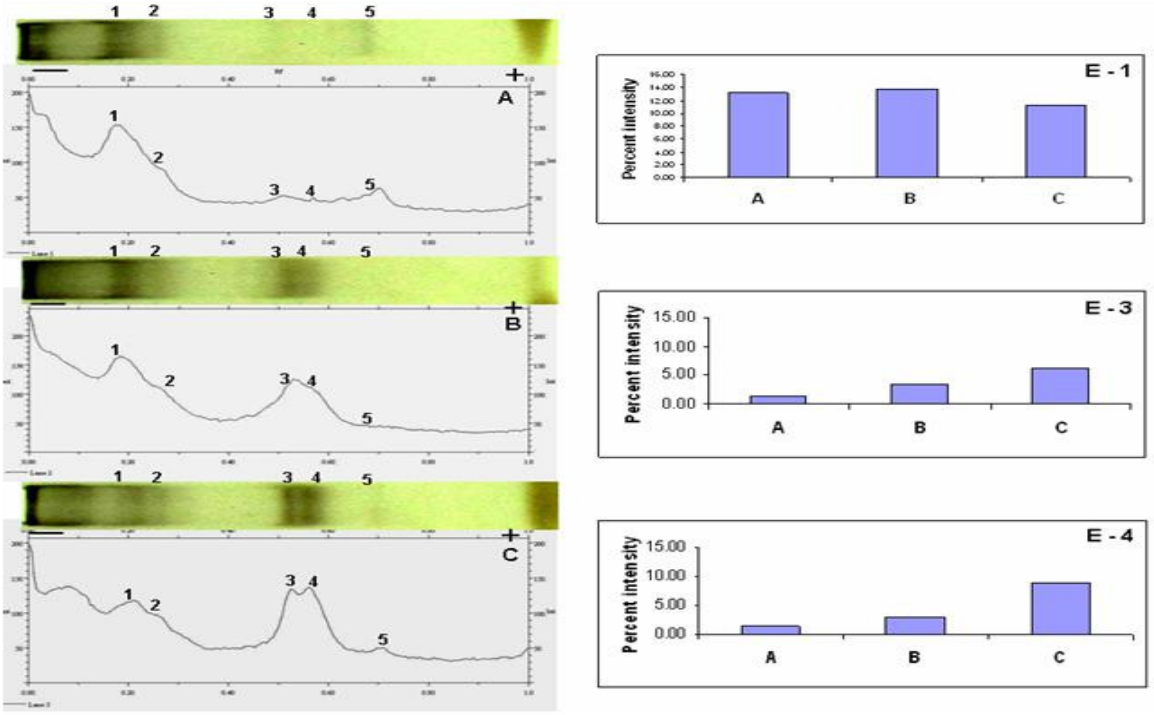

Figure-3

Isoforms of Esterase in three developmental stages (non regenerating callus - A, regenerating callus – B, leaves of regenerated plantlets – C) and their respective densitometry scanning; respective band / peak is numbered of which three significant isoesterases (E-1, E-3 and E-4) showing relative up- down regulation (based on percent intensity) in different developmental stages

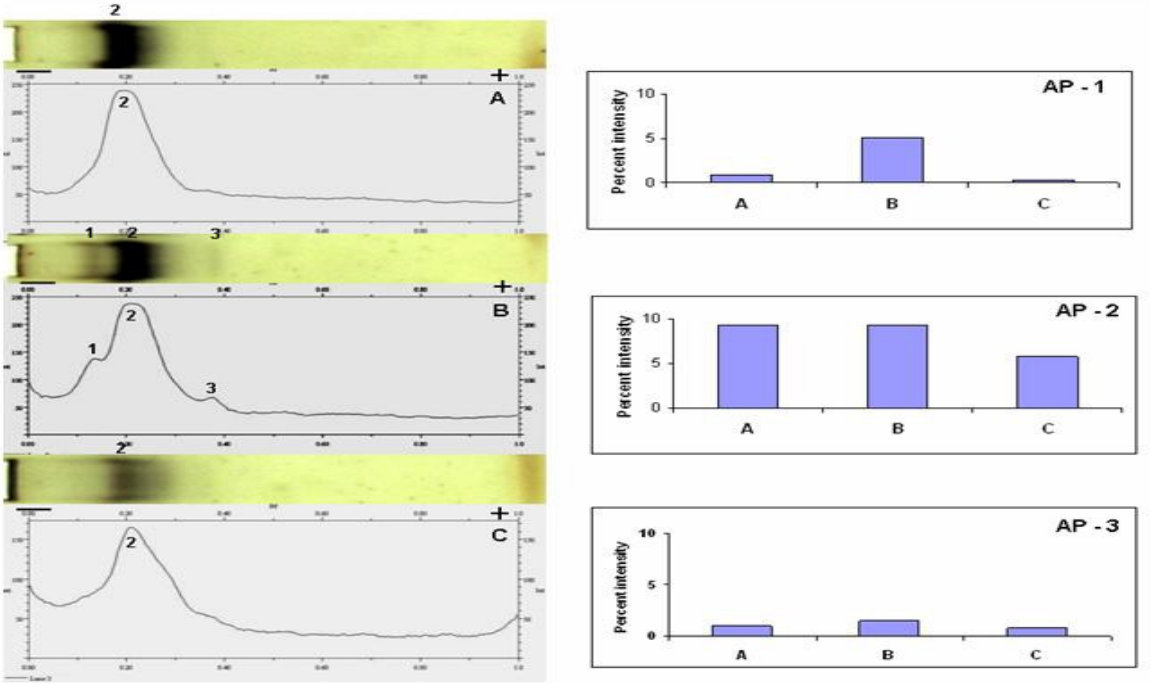

Figure-4

Isoforms of Acid Phosphatase in three developmental stages (non regenerating callus - A, regenerating callus – B, leaves of regenerated plantlets – C) and their respective densitometry scanning; respective band / peak is numbered of which three significant isoacid phosphatases (AP-1, AP-2 and AP-3) showing relative up- down regulation (based on percent intensity) in different developmental stages

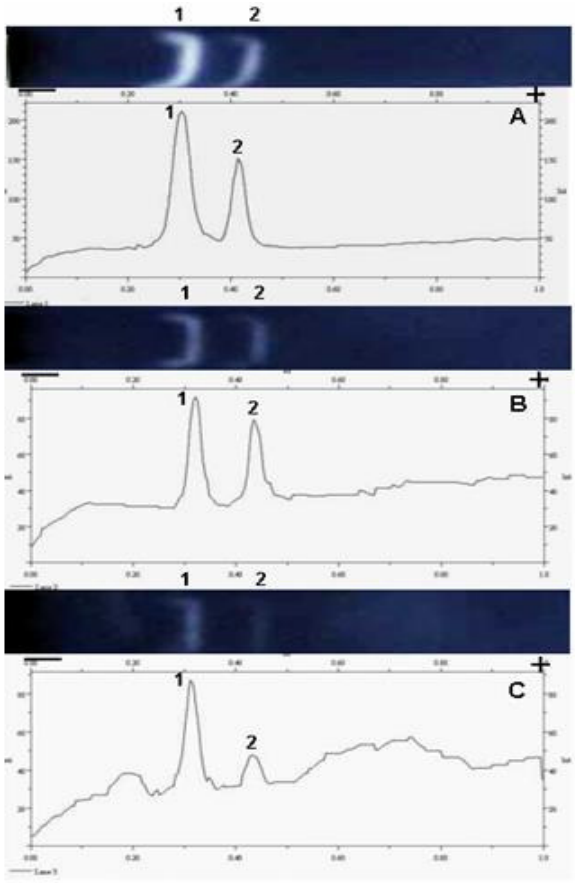

Figure 5

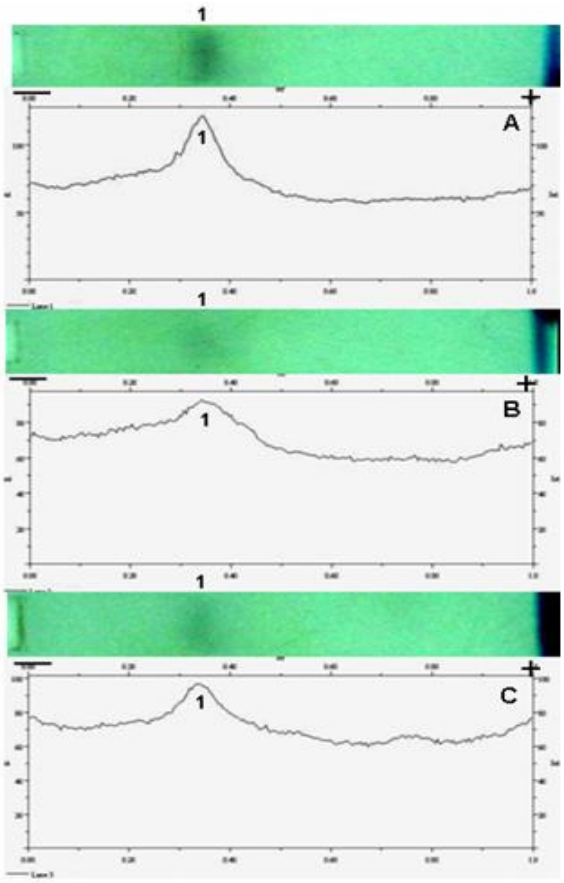

Figure 6

Figures-5-6

Isoforms of α–Amylase (Fig. 5) and Malate Dehydrogenase (Fig. 6) in three developmental stages (non regenerating callus - A, regenerating callus – B, leaves of regenerated plantlets – C) and their respective densitometry scanning; respective band / peak is numbered

Conclusion

Isozyme patterns are helpful tools for a better understanding of the basic mechanisms of cellular differentiation and further plant development. Tissue culture techniques allow the application of isozyme analysis to the morphogenic process, because they are endowed with relatively high amounts of plant material in the desired developmental stage. The present study with three *in vitro* developmental stages of tobacco has identified certain isoforms of Peroxidase, Esterase and Acid Phosphatase, which are either up- or down regulated in the course of dedifferentiation. The information, in the long run, will pave the way for precise functional genomics or proteomics based reverse genetics approach to look for the interplay of structural and regulatory genes governing plant morphogenesis.

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