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Looking for Novel Proteins Associated With *In Vitro* Morphogenesis of Pineapple

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Abstract

Pineapple [*Ananus comosus* (L.) Merr, family Bromeliaceae] is considered as one of the most economically important tropical fruits. Identification of novel proteins of this important fruit crop is an active area of research. Recent advances in plant biotechnology methods applied to pineapple crops have enhanced their potential application, both for basic studies and for direct application in agriculture. A hitherto not ventured direction is looking for enzymes and proteins related to *in vitro* morphogenesis, for which the present work was envisaged. Consequently, three distinct developmental stages (meristem with sheathing leaf base, regenerating callus and leaves) of *in vitro* regenerated and maintained pineapple plants were subjected to isozyme and proteomic analyses through one dimensional native activity gel and two dimensional gel electrophoresis respectively. 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. Two dimensional gel electrophoresis of total protein isolated from separate set of similar experimental materials revealed few unique protein spots after performing Peptide Mass Fingerprinting (MALDI-TOF).

1. Introduction

Pineapple is the second most important harvest crop worldwide. Nearly 70% of the pineapple produced is consumed as fresh fruit in producing countries. Identification of novel proteins of this important fruit crop is gaining momentum recently in following directions: characterization of Bromelain, a group of protein degrading enzymes

having immense clinical use (Secor Jr. et al. 2012); enzymes related to carbon fixation vis-à-vis photosynthesis since pineapple typically exhibits CAM metabolic pathway (Arago´n et al. 2013); and enzymes pertaining to fruit ripening (Moyle et al. 2005) to extend its shelf life.

Recent advances in plant biotechnology methods applied to pineapple crops have enhanced their potential application, both for basic studies and for direct application in agriculture (Gangopadhyay et al. 2005; 2009). Consequently, different *in vitro* developmental stages, obtained through standardized tissue cultural manipulations are amenable to specific isozyme and proteomic analysis, which otherwise would have been difficult using *ex vitro* samples.

In the backdrop of this, the present study was envisaged towards understanding the interplay of enzymes and proteins related to *in vitro* morphogenesis, a hitherto not ventured direction of protein identification in pineapple. Three distinct developmental stages (meristem with sheathing leaf base, regenerating callus and leaves) of *in vitro* regenerated and maintained pineapple plants were subjected to isozyme and proteomic analyses through one dimensional native activity gel and two dimensional gel electrophoresis respectively. The isozymes under study were Peroxidase (E.C.1.11.1.7), Esterase (E.C.3.1.1.1), Acid Phosphatase (E.C.3.1.3.2), α-Amylase (E.C.3.2.1.1) and Malate Dehydrogenase (EC 1.1.1.37). Simultaneously two dimensional gel electrophoresis of total protein was isolated from separate set of similar experimental materials for identification of unique protein spots after performing Peptide Mass Fingerprinting (MALDI-TOF)

2. Experimental and Observations

2.1 Plant materials and Methods

An *in vitro* maintained pineapple (var. Queen) system was used for the present study. The developmental stages used are: regenerating calli persistently giving rise to plantlets via somatic embryogenesis, meristem with sheathing leaf base and leaves. The last two samples were harvested from the regenerated plantlets after thirty days of subculture in respective media. Extraction for crude protein containing the enzymes was done using 1 g of fresh samples in each developmental stage. Samples were homogenized at 4°C with 1.5 cm3 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. 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 with little modifications (Sultana and Gangopadhyay 2014). For two dimensional gel electrophoresis total protein was extracted from isolated using ReadyPrepTM Protein Extraction Kit of *BIO-RAD*. Isolated protein was subsequently quantified by *RC DC* protein Assay kit and purified by ReadyPrepTM 2-D Cleanup

kit of *BIO-RAD*. The protein solutions (containing 100 µg protein) were loaded on dry IPG strips with re swelling buffer of pH gradient 3 to 10 NL (Immobiline dry strip, 13 cm, GE Healthcare). The strips were then re hydrated overnight, and isoelectric focusing was performed according to the following voltage gradient: 50 v for 4 h, 50 to 500 v for 30 min, 500 v for 2 h, 500 to 2000 for 1 h 30 min, 2000 v for 2 h, 2000 to 3500 for 1 h and finally 3500 v for 11 h for a total 38000 volt hours. Equilibration of gel strips was performed in an SDS equilibration buffer with DTT for 15 min and then with iodoacetamide for 15 min. Second dimension gel electrophoresis was performed on 10% PAGE followed by standard protocol of silver staining method for detection of protein spots. Gel images were digitized with a Bio-Rad Versa Doc. Spot of interest was subjected to MALDI-TOF for Peptide Mass Fingerprinting.

2.2. Results.

The five isozymes showed differential banding profiles in activity gels. Of the five, α -Amylase and Malate Dehydrogenase showed little alteration in activity or alteration in banding pattern in three developmental stages under study. Peroxidase, Acid Phosphatase and Esterase isozymes, however, revealed significant up or down regulation in the course of dedifferentiation. Furthermore, few developmental stage specific isoforms were also noticed (in Peroxidase and Esterase), which need further investigation to warrant their credibility as developmental stage specific isozyme marker(s).

Proteomic analysis through 2-dimensional gel electrophoresis further revealed certain developmental stage specific expression of few unique spots. These spots showed significant homology with development stage related proteins in database. Validations of these protein spots are underway both through MS/MS (MALDI-TOF/ TOF) spectrometry and reverse genetics approach.

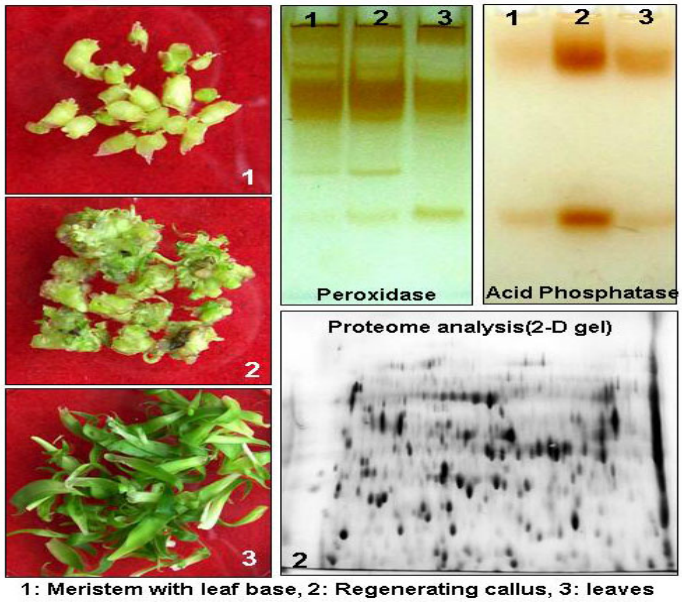

Figure1. Representative images of isozyme and proteomic analysis of three different in vitro developmental stages of pineapple

3. Conclusions

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 pineapple has identified certain isoforms of Peroxidase, Esterase and Acid Phosphatase, which are either up- or down regulated in the course of dedifferentiation. The information will supplement proteomics based approach to look for the interplay of structural and regulatory genes governing plant morphogenesis.

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