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Elicited Phenyl-Propanoid Metabolism in Hairy Root Cultures of Tomato

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ABSTRACT

HCHL gene encoding p-Hydroxycinnamoyl-CoA hydratase/lyase from *Pseudomonasfluorescens* AN103 was expressed in leaves of *Lycopersiconesculentum*. *Agrobacteriumrhizogenes* was the vector in gene transfer. Initiation of Hairy roots was observed in two to three weeks from the transformed sites of mature Tomato leaves. In an effort to study the expression and exploit the ability of HCHL gene the activity of enzyme PAL(Phenylalanine ammonia lyase) gained its importance since its activity in-turn influence the activity of HCHL enzyme which plays a vital role in the formation of flavonoid, Vanillin. In the present study the fungus, *Rhizopus* was used as an elicitor to elicit the PAL activity in Hairy root cultures of Tomato. In the first 24hrs. of elicitation it was found that the activity of PAL was increased. Later a decrease in the activity was noticed.

Keywords: p-Hydroxycinnamoyl-CoA hydratase/lyase (HCHL), Phenylalanine ammonia lyase (PAL), *Pseudomonas fluorescens* AN103, *Agrobacteriumrhizogenes, Lycopersicon esculentum, Rhizopus,* Vanillin

INTRODUCTION

Phenylalanine ammonia lyase(PAL) is an enzyme which forms an interface between an aminoacid, Phenylalanine and the Phenylpropanoid metabolism It catalyses a non-oxidative deamination of Phenylalanine to form the first secondary phenylpropane structure, Ethyl(E)-cinnamate. The final step of the general Phenyl propanoid metabolism is the carboxyl activation of hydroxyl-cinnamate: CoA lyase which on further condensation with malonyl-CoA lead to formation of flavonoids.

Vanillin formation from Vanillic acid is often interfered by the formation of Protocatechuic acid but the enzyme Hydroxycinnamoyl-CoA re-routes the phenyl propanoid metabolism. A construct containing the gene HCHL of bacterial origin wasexpressed in *Lycopersicumesculentum* of family Solanaceae.

Some algae like *Nostoc*, fungi like *Aspergillus*, stress or some precursors like Ferulic acid, phycocyanins etc., elicits the catalytic action of PAL which in-turn influences the expression of the gene HCHL. So an effort was made to use the fungus, *Rhizopus* as an elicitor to re-route the Phenylproponoid pathway and produce the glycosides of interest, especially Vanillin by enhancing the activity of PAL.

MATERIALS AND METHODS

Geniticallyengineered Ri plasmid of *Agrobacterium rhizogenes*harbouring HCHL gene encoding an enzyme 4-Hydroxycinnamoyl-CoA hydratase/lyase from *Pseudomonasfluorescens* AN103 is used to Bio-transform the Mature leaves of *Lycopersicumesculentum* . In-vitro elicitation of phenyl-propanoid pathway is done by co-culturing *Rhizopus* within the suspension cultures of Hairy roots of *L. esculentum.*

Initiation and establishment of Hairy Root cultures:
Surface sterilized mature, clean, disease free leaves by treating with 70% ethanol for 1min. and 0.1N $HgCl_2$ for 15min. and rinsed thoroughly withdistilled H_2O to remove traces of $HgCl_2$.Trimmed to desirable size. Wounded the veins and injected *A. rhizogenes* as wounded cells are the sites of DNA transfer by *A. rhizogenes* which was initially sub-cultured and grown for 48hrs. in YMB medium(5ml.) with antibiotics Ampicillin(12.5µl/5ml) and Kanamycin(12.5µl/5ml). Blotting of excess *Agrobacterium* was done to avoid excessive growth of bacteria in culture media. Explants were inoculated in Sucrose containing MS Agar slants with phytoharmones adjusted to pH(5.6-6.0) at 27^0 C.

Micro scale establishment of Hairy root cultures was done in Murashige and Skoog Rooting Media devoid of growth regulators, pH (5.6-6.0) and incubated for 2 weeks to 3 weeks aerobically. Excised root tips sub-cultured in MS Rooting media with antibiotics Ampicillin and Kanamycin were screened. High density cultures were obtained by culturing in B_5 media with high Ampicillin (250µg/ml) at pH (7.0)

Harvest of Hairy roots of Tomato:
Harvested the hairy roots(2 weeks old) from the medium washed 3 to 4 times with H_2O. Dried completely with filter paper. Weighed the sample. Wrapped it in aluminium foil, dipped in liquid N_2 stored in plastic packets at -20^0 C or -70^0 C for further use.

Preparation of elicitor:
Rhizopus was grown for 15-21 days in MS(1/2x) broth under static conditions at 31^0 C.Autoclaved the fungal culture and filtered the culture media. Washed the mycelial mat several times with distilled water. Homogenized the mycelia in motor and pestle. Made an aqueous extract and autoclaved. Stock cultures were maintained at 4^0 C.

Elicitation of Hairy root cultures:
Week old Hairy roots were weighed in aseptic conditions.Treated with elicitor(1ml/1mg). Control was treated only with distilled water.

Quantified PAL activity and phenolics (soluble and cell wall bound after 24hrs. of elicitation)

Estimation of PAL activity:
Homogenized Hairy roots to powder by taking liq. N_2 and a pinch of polyclar VT. Added minimum amount of extraction buffer(Tris-HCl 100mM), Na_2EDTA(10mM), DTT(20mM) at pH(8.5) and left at 4^0 C for 15 to 20 min. Centrifuged and de-salted by dialyzing agent or extraction buffer and left over-night at 4^0 C. The dialyzed sample was used as an enzyme source and treated for PAL activity.

PAL Assay:
Performed by taking Control containing 150µl enzyme extract, 850µl assay buffer and Sample containing 150µl enzyme extract, 650µl Assay buffer(Tris-HCl 100mM, pH-8.5), 200µl Phenylalanine.

Characterization of PAL activity:
Characterized PAL activity at O.D 270nm in UV-Vis Spectrophotometer(Systronics-117). Inhibition of the reaction was done with acidified MeOH (3:1 Acetic acid: Methanol) and concentrated assay mixture in Rotary Vacuum Evaporator and loaded on TLC.

Quantification ofSoluble and Cell wall bound Phenolics:
Ground the tissue into powder by liq N_2 and added methanol (80% v/v), left undisturbed for 1hr. and centrifuged.. The supernatant is the source of soluble phenolics and the pellet for cell wall phenolics. The supernatant was concentrated in Rotary Vacuum Evaporator and re-suspended in aqueous Methanol(50% v/v)

Cell wall preparation:
The pellet was consequently treated with following solvents (1M NaCl 0.5%), aqueous SDS(0.5%), MeOH(3x), water(2x), acetone(2x), di-ethyl ether) as suggested[3] and homogenized for 15 to 30min. Centrifuged for 5min. at 10,000rpm. The remaining insoluble material is purified cell wall which was dried overnight and processed further.

Alkali Treatment:
To the dried powder added 2M NaOH(15mg powder/4ml of 2M NaOH) and kept in dark for 24hrs. Filtered and acidified to pH-2.0. Ethyl acetate twice the volume of supernatant was added. The supernatant expected to contain the phenolics is separated and concentrated in Rotary Vacuum Evaporator and re-suspended in aqueous Methanol(50% v/v) and loaded on TLC made of Silica gel F_{254} and developed in a saturation chamber containing acetone: $CHCl_3$:Formic acid in a ratio of 5:4:1 and viewed under UV lamp for identification against the authentic standards Phenyl alanine, Cinnamic acid, Ferulic acid and Vanillin.

RESULTS AND DISCUSSION

Initiation of hairy roots from transformed cells irrespective of dorsal or ventral side of mid-vein or lateral vein approximately in 2 to 3 weeks after inoculation of explant. Root induction is associated with random insertion of T-DNA to the chromosomal DNA of *Lycopersiconesculentum* The interest in the present investigation is the expression of 4-Hydroxycinnamoyl-CoA Hydratase/lyase(HCHL), an enzyme of Phenyl propanoid chain cleavage characterized from *Pseudomonasfluorescens* strain AN103[1][2]. Rapid and incessant growth of Hairy roots confirms the transgenesis.

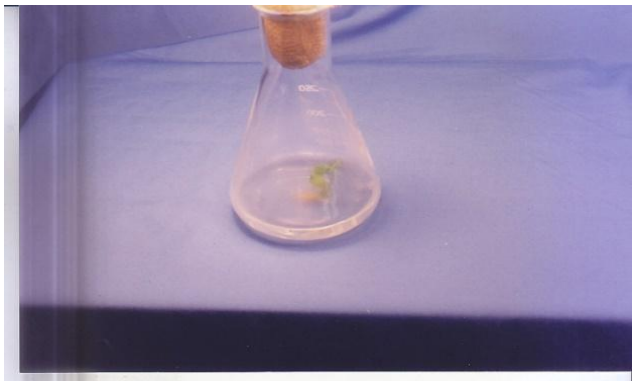

Fig. 1: Transgenic *Lycopersicum esculentum* showing Hairy roots

No soluble phenolics were detected in MeOH fraction. Cell wall bound phenolics were detected to some extent showing the presence of Phenyl alanine, hydroxycinnamate trans-cinnamic acid.

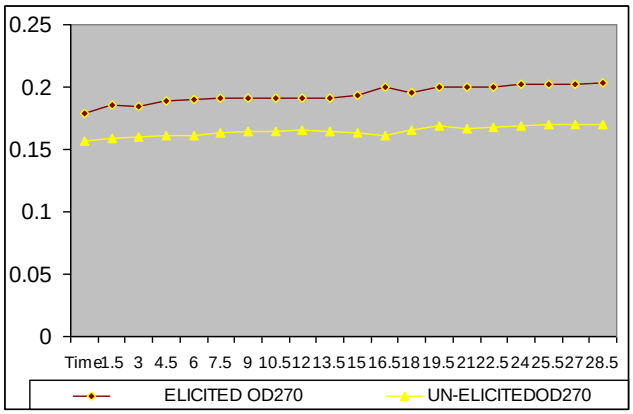

Fig. 2: PAL activity of unelicited and elicited Hairy Root cultures of *L. esculentum*

PAL activity showed a considerable increase in a time interval of 24hrs. elicitation analysed at every 1.5min. interval. Later as time progressed decrease in PAL activity was noticed.

CONCLUSION

Initiation of the hairy root cultures in the mature leaves of Tomato indicates the expression of HCHL gene to be positive and *L. esculentum* has been genetically modified. Activity analysis of PAL shows that *Rhizopus* acted as a source of elicitation in root organ cultures.

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