ISOPENTENYL TRANSFERASE GENE INCORPORATED GM RUBBER- *EFFECT* ON PLANT REGENERATION.

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Objective:

To regenerate *Hevea* transgenics with isopentenyl transferase gene incorporation through *Agrobacterium tumefaciens* mediated genetic transformation.

Significance in *Hevea*

- **In *Hevea* high cytokinin levels have been reported earlier in TPD tolerant plants.**
- **IPT gene incorporation may help in reducing the severity of TPD syndrome.**
- **Enhanced vegetative growth**

IPT GENE

This is a bacterial gene present in the *tmr* locus of *Agrobacterium* which catalyses the first step in cytokinin biosynthesis.

Hormone levels in plants could be varied rapidly using this system.

Changes in gene expression occurring rapidly in response to endogenous hormone fluctuations can be noticed accurately.

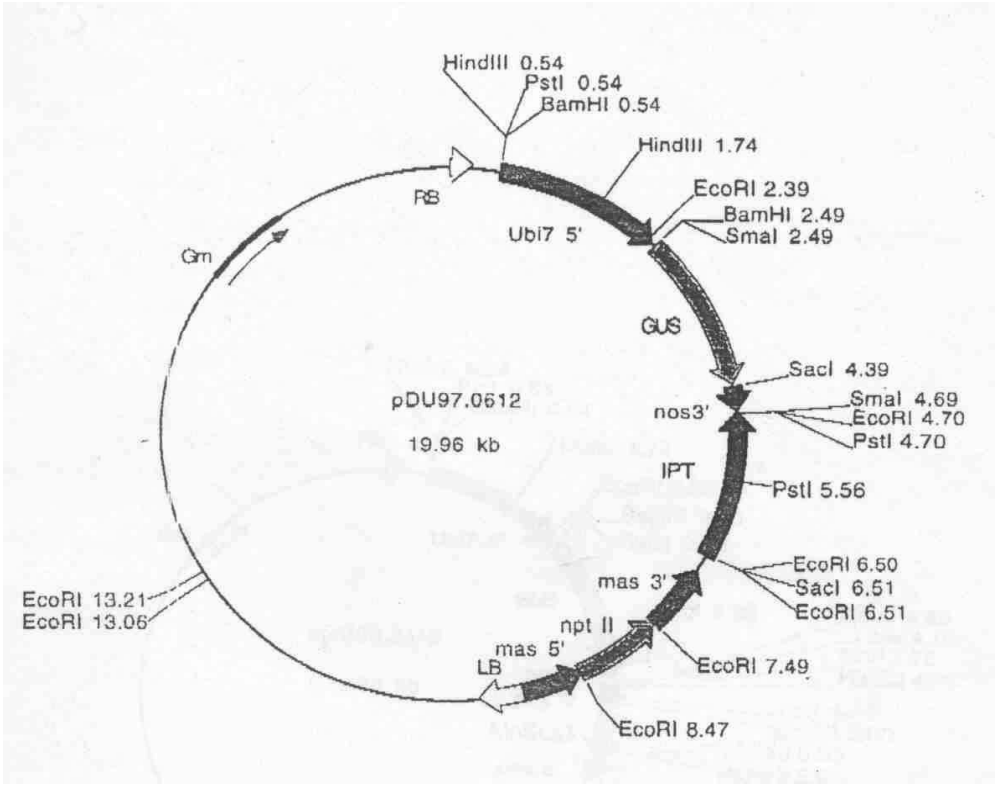
Significance - ipt transgenics

According to earlier reports, *ipt* transformed plants usually show –

1. Hormone autotrophic growth due to high cytokinin/auxin levels.
2. Enhanced vegetative and reproductive growth.
3. Increased photosynthetic rate associated with enhanced stomatal conductance.
4. Reduced seed dormancy and leaf senescence.
5. Enhanced nitrate reductase activity.

Plasmid Expression Vector for Ipt gene

Plasmid vector used was PDU 97.0612 which contained GUS as the reporter gene and npt II gene for selection in plant cells. Plasmid also contains the nucleotide sequence for *ipt* gene from *tmr* locus of *Agrobacterium* under the control of it's own promoter.



Materials and Methods

Target tissue regeneration

Callus was induced in leaf cultures of clone RRII 105. Through repeated subculture in proliferation medium by gradually increasing cytokinin/auxin ratios and induction of stress the callus was made embryogenic. Embryogenic callus was used as target tissue for *Agrobacterium* infection.

***Agrobacterium* culture preparation**

Agrobacterium tumefaciens strain EHA 101 harboring the plasmid vector PDU 97.0612 were incubated overnight at 28°C in AELB liquid medium containing the antibiotics gentamycin 20 μg/ml and kanamycin sulphate 50 μg/ml

OD of the culture was measured and volume was adjusted to get a bacterial density of 5×10^8 cells/ml.

The culture residue was diluted in induction medium containing 100 μM acetosyringone and 1.0 mM each proline and betaine HCl.

The culture was activated for four hours and used for tissue infection.

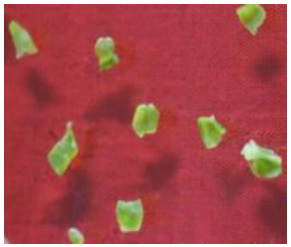
Transformation methodology

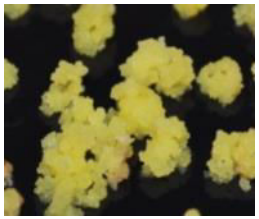
The following parameters influencing effective transformation were standardized.

- Target tissue – Leaf embryogenic calli
- Duration of infection - 15 min.
- Duration of co-culture - 3 days
- Frequency of subculture - 3 weeks interval
- Pre selection medium - Cefotaxime 500 mg/l
- Selection medium - cefotaxime 500 mg/l & kanamycin 300 mg/l

Hevea genetic transformation and transgenic plant regeneration - Protocol

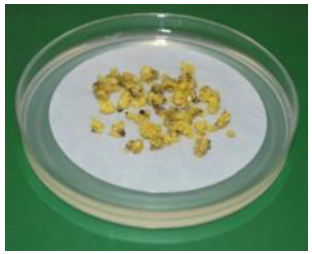
Explants





Hevea Callus

Bacterial Infection

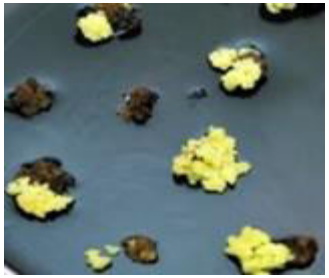


Bacterial co-culture

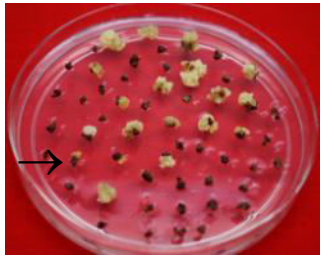
callus

embryos

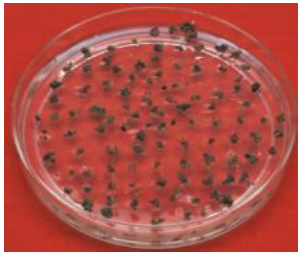
GUS expression



Transgenic callus



Transgenic cell lines



Control callus

Transgenic embryos

Transgenic plant

Hardened transgenic plant

Selection of transformed lines

- After one week culture in pre selection medium, the tissues devoid of any bacterial over growth were transferred to selection medium and subcultured every 20 days.
- Putatively transformed cell lines screened for GUS activity and positive ones were periodically subcultured every 30 days in fresh medium.
- After 3 months, antibiotic concentration was reduced to 100 mg/l kanamycin.

Embryo induction, maturation and plant regeneration.

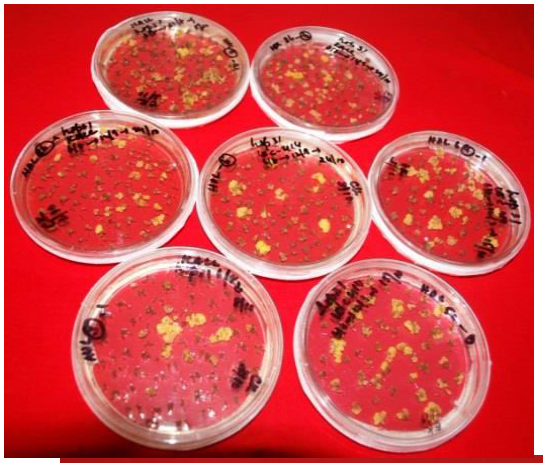
- Somatic embryogenesis was obtained when proliferated callus was subcultured in embryo induction medium containing phytohormones and stress inducing compounds such as poly ethylene glycol (8 g/l) and enhanced phytagel (0.3 - 0.5%).

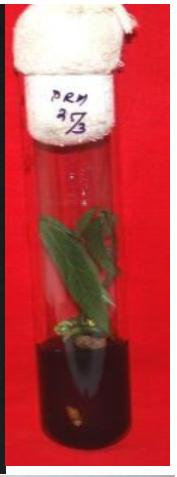
- Different levels of the hormones BA (1.0 mg/l), Kin (2.0mg/l) and GA_3 (0 -1.0 mg/l) in presence of IBA (0.8 mg/l) were tried for plant regeneration.

- Plant regeneration was obtained in modified Murashige & Skoog (MS) medium supplemented with organic supplements such as malt extract (200 mg/l), coconut water (10%) and phytohormones GA_3 , BA and IBA.

ipt gene incorporated *Hevea* Transgenic plant regeneration &acclimatization

Transgenic plants of clone RRII 105 incorporated with ipt gene for enhanced cytokinin synthesis were developed *in vitro* and are being acclimatized.

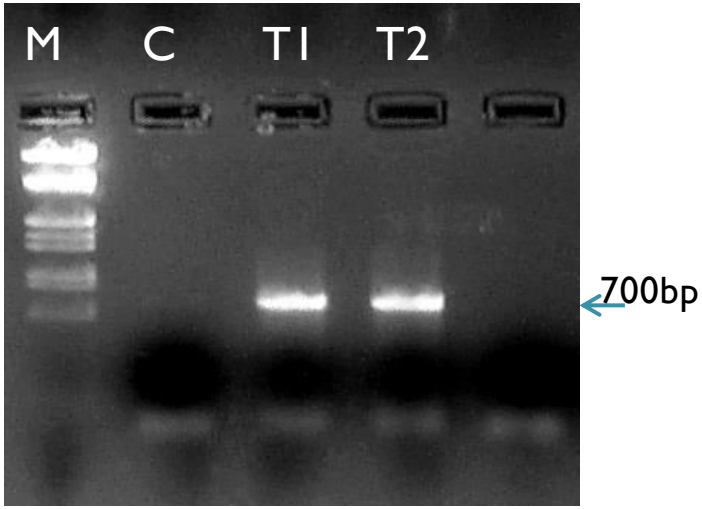





Morphological Abnormalities in regenerating plants

PCR Analysis

- PCR amplification was carried out using isopentenyltransferase gene specific primers. Since *ipt* is a bacterial gene responsible for cytokinin biosynthesis, *ipt* primers were designed from the coding region sequence of isopentenyl transferase gene sequence of *Agrobacterium tumefaciens.*
- Forward primer: 5' CTTGCACAGGAAAGACGTCG3'
- Reverse primer: 5' CGTAAGCGGCTGCG3'
- The PCR products were analysed in 1.5 % agarose gels.
- An amplified band of approximately 700 bp was obtained in the transgenic callus showing gene integration.



Conclusion

- Transgenic plants incorporated with ipt gene are expected to have enhanced vegetative growth and TPD tolerance

Thank you