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# *Hevea* GENETIC TRANSFORMATION FOR ENHANCED RECOMBINANT PHARMACEUTICAL PRODUCTION BY THE USE OF HEVEIN PROMOTER (02 03 12 SF0009)

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## INTRODUCTION

Genetic engineering paves the way to convert plants into factories that produce commercially useful products. Such production of recombinant proteins and biomolecules in plants is an emerging field of transgenic research, popularly known as "Molecular Farming". The effective use of plants as bioreactors requires sustained high level production that is stable over the lifetime and in subsequent generations at a relatively low cost

At the Rubber Research Institute of Malaysia (RRIM), an *Agrobacterium tumefaciens*-mediated genetic transformation procedure for *Hevea brasiliensis* cultivar GL 1 was established using anther callus. In the past, the RRIM has generated transgenic rubber plants that express a bacterial enzyme (β-glucuronidase or GUS), a single-chain variable-fragment (scFv) antibody against the coat protein of *Streptococcus gordonii*, an oral dental bacterium and human serum albumin - a protein of commercial value, in the latex cytosol. The transgenic *Hevea* expressed recombinant human serum albumin in concentrations of up to 0.3% of the total soluble latex protein, while the scFv in latex serum was estimated at 0.02% of the total protein; these figures are comparable to estimates of 0.01-1.0% foreign proteins reported in other transgenic plants.

Despite the significant cost advantage in using plant as hosts, recombinant protein production rates in transgenic plants, including *Hevea*, needs to be significantly increased to be competitive with conventional bioreactor systems. The expression of human serum albumin from *Pichia pastoris*, for example, was reported at ca 150 mg/l; this is 4.5 times of that obtained in the latex serum of transformed *Hevea*. One way to increase recombinant protein production in plants is by manipulating the structure of gene constructs employed in genetic transformation. In *Hevea* - the hevein promoter - which is a strong latex specific promoter, was sought with the aim to improve the production of recombinant proteins in the latex cytosol. Both the hevein gene and its promoter sequence have now been isolated and characterized. In an earlier study, constructs bearing the hevein promoter fragments; HevP1 (0.35kb), HevP2 (0.45kb), and HevP3

(0.73kb) fused to the *uidA* reporter gene in the pGPTV-Kan vector have shown to induce GUS expression. In the present work, the *uidA* gene for the said constructs is replaced with the pharmaceutical genes, Human Protamine 1 (HP1) and Human Atrial Natriuretic Factor (HANF) in order to evaluate their expression in the transformed *Hevea*.

## MATERIALS AND METHODS

### Cloning of Human Protamine 1 (HP1) and Human Atrial Natriuretic Factor (HANF) cDNAs

The plant transformation vectors (pGPTV-Kan) fused with the hevein promoter fragments and *uidA* reporter gene that were employed in an earlier experiment were selected for the insertion of the HP1 and HANF genes. The vectors contain kanamycin resistance gene (*npt*II) as the plant selectable marker gene. IMAGE CLONE 1257590 containing the HP1 cDNA and IMAGE CLONE 306592 containing the HANF cDNA were obtained from HGMP Resource Centre, UK. The HP1 and HANF genes were amplified using the primers designed from the HP1 and HANF cDNAs sequence respectively. The amplified fragments were double digested with *Sma*1 and *Sac*1 restriction enzymes (New England Biolabs, MA, USA). The three constructs (pGPTV-HevP1, -HevP2, and -HevP3) were also restricted using the same enzymes in order to remove the *uid*A reporter gene. The vector fragments devoid of the *uid*A gene were isolated from the agarose gel and purified using the QIAquick Gel Extraction Kit (QIAGEN, CA, USA). The HP1 cDNA (0.2kb) and the HANF cDNA (0.45 kb) were ligated with the three vector fragments. The ligation products were then transformed into DH5α *Escherichia coli.* The construct harbouring the pharmaceutical gene (HP1 or HANF) in the correct orientation was then electroporated into *A. tumefaciens* GV2260 strain using the Gene Pulsar electroporator (Bio-Rad Laboratories, CA, USA). The transformed GV2260 cells were grown on the LBA plates containing kanamycin (100µg/ml) and rifampicin (100µg/ml).

### Co-cultivation of *Hevea* Anther Callus with *Agrobacterium* Culture

*A. tumefaciens* GV22260 that harbour pGPTV-HP1-HevP1, pGPTV-HP1-HevP3, pGPTV-HANF-HevP1, pGPTV-HANF-HevP2 and pGPTV-HevP3 were grown on LB liquid media containing kanamycin (100µg/ml) and rifampicin (100µg/ml) overnight at 28˚C. The $OD_{600nm}$ of the bacterial suspension culture was adjusted accordingly to approximately 0.5 using CI media.

The anther cell wall of GL1 cultivar were grown on MS (ID)Z media to induce callus formation. The callus culture was then co-cultivated with *A. tumefaciens* harbouring the desired genes to allow their insertion into rubber genome. After co-cultivation, the calli were transferred to fresh initiation medium containing cefotaxime, ticarcillin and kanamycin to eliminate overgrowth of *Agrobacterium*, while kanamycin is used to screen the calli that has taken up the desired genes. The plates were incubated in the dark at

25˚C for 14 days (first selection); typically, the cultures normally undergo ten rounds of selections prior to embryogenesis.

**PCR Analysis of *Hevea* Transformants**

Genomic DNA was extracted from putative positive callus, and leaf tissue sampled from the regenerated plantlets using the plant DNA extraction kit (QIAGEN, CA, USA). The presence of the inserted genes was analyzed using different sets of primers, *i.e.* FP.hpheveinpro and RP.hpheveinpro for confirmation of HP-1; FP.CDP1 and RP.CDP2 for confirmation of HANF; while FP.hevP3 and RP1.hevP3, RP2.hevP3 or RP3.hevP3 were employed for determination of hevein promoter fragment sequences. The plant selectable marker gene (*npt*II) was amplified using the FP.nptII and RP.nptII.

## RESULTS

**Human Protamine 1 (HP1) and Human Atrial Natriuretic Factor (HANF) gene constructs in pGPTV-Kan Expression Vector**

The HevP-(PHev2.3) hevein promoter template that was isolated in a previous work was used to generate three overlapping fragments of decreasing length in the upstream region (HevP-3, HevP-2 and HevP-1). In a preliminary experiment, these fragments were fused to ß-glucuronidase (*uid*A) reporter gene in the pGPTV-Kan vector and three constructs were generated; pGPTV-Kan-1, pGPTV-Kan-2, pGPTV-Kan-3); pGPTV-Kan contains neomycin phosphotransferase (*npt*II), a selectable marker which is located near the T-DNA left border, while *uid*A, the reporter gene is located near the right border, and unique restriction sites. In order to reduce antisense effects, the reporter and selectable marker genes have been arranged in a divergent orientation. The plant selectable marker genes *npt*II is driven by the nopaline synthase (*nos*) promoter; and the terminator sequences at their 3' terminus to ensure that transcription ceases at the correct position. The pGPTV vector is a derivative of the plant binary vectors pBIN19, pBI101 and pBIB-HY6. The vector also contains the broad host range RK2 origin of replication and a bacterial kanamycin resistance marker for growth in *E. coli* and *A. tumefaciens*.

In the present study, the *uid*A gene in the genetic constructs was replaced with HP1 or HANF pharmaceutical genes; the resulting pGPTV-HP1-HevP1, pGPTV-HP1-HevP2, pGPTV-HP1-HevP3, pGPTV-HANF-HevP1, pGPTV-HANF-HevP2 and pGPTV-HANF-HevP3 vectors were initially cloned into *E. coli* DH5α. Nucleotide sequence confirmed that the HP1 or HANF constructs were inserted in the correct orientation in relation to the hevein promoter fragment sequences. The confirmed gene constructs were then electroporated into *A. tumefaciens* GV2260 strain containing the supervirulent plasmid pToK47, which in previous studies has shown to improve the efficiency of transformation.

**Genetic Transformation of *Hevea* GL 1 with Human Protamine 1 (HP1) and Human Atrial Natriuretic Factor (HANF)**

Co-cultivation of the GV2260 bearing HP1 and HANF genes was performed with GL1 anther callus culture. In this experiment, pLGMR.HP1, pBINPLUS-HANF and pGPTV.ScFv4715 constructs served as positive controls. The growth of the putative transformant callus was monitored on a growth media containing kanamycin as a selection marker. The kanamycin resistant-callus underwent a series of sub-culturing and multiplication. At this stage, PCR analyses of genomic DNA extracted from a number of putative HP1 and HANF transformants were performed to detect the inserted genes; FP.hpheveinpro and RP.hpheveinpro primers were used to amplify HP1 gene, while the HANF gene was detected using FP.CDP1 and RP.CDP2 primers. FP.hevP3 and in combination with RP1.hevP3, RP2.hevP3 or RP3.hevP3 primers were used to determine the presence of the hevein promoter fragments. The remaining callus tissues were sub-cultured and transferred into differentiation media for embryoids formation and plantlet regeneration. Putative transformed plantlets were obtained for HevP1-HP1, HevP3-HP1, HevP1-HANF, HevP2-HANF, and pBINPLUS-HANF; PCR analysis performed on leaf genomic DNA samples confirmed the presence of the transgenes. The transformed plantlets were then transferred into polybags and maintained at the contained plant house at the RRIM Research Station to verify expression of transgenes in the latex cytosol.