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Genetic Transformation of Hevea brasiliensis *with Human Atrial Natriuretic Factor*

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Agrobacterium mediated genetic transformation was performed on anther callus of Hevea *cultivar GL1 with a gene encoding human atrial natriuretic factor (HANF), a peptide-hormone that is involved in regulating cardiac blood pressure. The gene constructs containing four different lengths of the indigenous hevein promoter, also incorporated the marker* nptII *for selection of transformants. Thus far 28 plants were regenerated and their bud-grafted generations were successfully transferred to the soil; all transformants tested positive for the promoter-transgene inserts. The presence of the HANF transcript in leaf samples was detected by RT-PCR in a number of original transformants and their vegetative generations, and the authenticity of the amplicon was confirmed by nucleotide sequencing. Polyclonal anti-atrial natriuretic peptide detected a band in the low molecular weight region in Western-immunoblot of leaf protein of an original transformed plant, while MALDI TOF-TOF and database searches of the corresponding protein band on SDS-gel revealed matches to atrial natriuretic peptide in the C-terminus of HANF.*

Keywords: *Hevea brasiliensis*, genetic transformation, human atrial natriuretic factor, RT-PCR, protein mass-spectra analysis

The Rubber Research Institute of Malaysia (RRIM) developed an *Agrobacterium* mediated anther callus genetic transformation procedure for *Hevea brasiliensis* cultivar GL1[1]. In the past, RRIM regenerated transgenic rubber plants that expressed 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 expression of human serum albumin and the scFv in latex serum of the transgenic rubber plants were at levels comparable to the estimates (0.01–1.0% of total protein) of recombinant proteins reported in other transgenic plants that are being developed as bioreactors[2]. In this aspect, *Hevea* has an obvious advantage as the plant's laticiferous system offers sustainable production and continual harvest of recombinant proteins over the lifetime of the transformant and in subsequent vegetative generations[3]. Even so, recombinant protein production in transgenic plants needs to be improved so that it is comparable to that

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of a microbial system (typically, yeast and bacteria)[4].

Generally, the CaMV 35S 'universal' promoter is employed to drive transgene expression in *Hevea*, which results in recombinant proteins synthesis, albeit at low levels, in the latex and many other tissues of *Hevea*[5]. Therefore, we have adopted the popular strategy to enhance transgene expression in plants *i.e.* by manipulating the structure of gene constructs employed in genetic transformation. In this case, a latex specific promoter, the hevein promoter was chosen simply because the native hevein protein is the most abundant protein in the lutoids of latex. For this, both the hevein gene and its promoter sequence have been isolated and characterised[6]. In a recent study, constructs bearing the hevein promoter fragments; HevP1 (0.35 kb), HevP2 (0.45 kb), HevP3 (0.73 kb) and HevP4 (0.95 kb) fused to *uidA* (β-glucuronidase or GUS) reporter gene in the pGPTV-Kan expression vector have shown the ability to induce GUS expression in *Hevea* latex; the enzyme activity was relatively higher than that discerned in the earlier GUS transformants[7]. In the present work, the *uidA* gene for the said constructs is substituted with the Human Atrial Natriuretic Factor (HANF) cDNA, which encodes for a precursor protein that contains two peptides of pharmaceutical value[8] *i.e.* cardiodilatin peptide (N-terminus) and atrial natriuretic peptide (C-terminus)[9], in order to evaluate its expression in transformed *Hevea* plants.

MATERIALS AND METHODS

Preparation of Human Atrial Natriuretic Factor (HANF) Gene Construct

The *uidA* reporter gene that was employed in a recent study was excised from the plant transformation vectors fused with the four different lengths of hevein promoter fragments (pGPTV-Kan-hevP1, pGPTV-Kan-hevP2, pGPTV-Kan-hevP3 and pGPTV-Kan-hevP4). The pGPTV-Kan vector contains neomycin phosphotransferase (*nptII*), a selectable marker which is located near the T-DNA left border, while *uidA* with unique restriction sites at extremities is located near the right border. In order to reduce antisense effects, the reporter and selectable marker genes have been arranged in a divergent orientation[10]. The plant selectable marker gene *nptII* is driven by the nopaline synthase (*nos*) promoter while the terminator sequences at their 3' terminus ensure that transcription ceases at the correct position[6]. The pGPTV vector is a derivative of the plant binary vectors pBIN19, pBI101 and pBIBHY6. The vector also contains the broad host range RK2 origin of replication and a bacterial kanamycin resistance marker for growth in *Escherichia coli* and *A. tumefaciens*[10].

The *E. coli* DH5α containing the HANF cDNA in pBluescript KS^+ vector (IMAGE CLONE 306592) was obtained from IMAGE Consortium, HGMP Resource Centre, UK. The HANF cDNA was amplified with ANF-for (forward) and ANF-rev (reverse) primers; the primers contain *Sma*I and *Sac*I restriction sites at their respective extremities. The HANF amplicon was then digested with *Sma*I and *Sac*I restriction enzymes (New England Biolabs, MA) to yield a 456 bp HANF cDNA that harbours its complete open reading frame. The four pGPTV-Kan vectors[6] were also digested with the same enzymes to remove the *uidA* reporter gene. The restriction digests were electrophoresed on agarose gel, the linearised vectors were then purified using QIAquick Gel Extraction Kit (QIAGEN, CA) and re-ligated with HANF cDNA at the compatible ends. The ligation products were then transformed into *E. coli* DH5α, and grown on LBA containing 50 µg/mL of kanamycin. The HANF gene in pGPTV-Kan vectors were detected by colony-

lysis PCR while nucleotide sequencing (First Base Laboratories, Malaysia) of the purified vectors confirmed presence of HANF cDNA. The HANF gene constructs in the PGPTV-Kan vectors are depicted in *Figure 1*.

The pGPTV-Kan-HANF vectors were then electroporated into *A. tumefaciens* GV2260 strain, along with pToK47 supervirulent plasmid (selectable with carbenicilin and streptomycin) that has been shown to improve transformation in *Hevea*[11], using the Gene Pulser electroporator (Bio-Rad Laboratories, CA). The electroporated GV2260 cells were grown on LBA plates containing kanamycin (50 µg/ml), rifampicin (50 µg/mL), carbenicilin (50 µg/ml) and streptomycin (50 µg/mL); the presence of HANF insert was detected by PCR, and glycerol stocks of each clone were stored in -70ºC until further use.

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

Large scale cultures of *A. tumefaciens* GV2260 that contained the four different HANF gene constructs were grown on LB broth containing kanamycin (50 µg/mL) at 28ºC until stationary phase. The OD_{600nm} of the bacterial culture was adjusted to *circa* 0.6 using culture initiation (CI) media. *Hevea* GL1 anther callus was initiated from the anther walls (tapetum cells) in MS (ID)Z media. The callus tissue was then co-cultivated with *A. tumefaciens* GV2260 harbouring HANF to allow infection and insertion of the desired transgene into the rubber genome as described elsewhere[5]. After co-cultivation, the callus tissue was transferred to fresh initiation medium containing cefotaxime and ticarcillin, to prevent overgrowth of *Agrobacterium*, while kanamycin in the selection media ensures growth of putative transformed callus. The plates were incubated in the dark at 25ºC for 14 days (first selection); the callus cultures were subjected to several rounds of selections prior to embryogenesis and subsequent regeneration of plantlets.

PCR and RT-PCR Analysis of *Hevea* Transformants

Genomic DNA was extracted from putative transformed callus and leaf samples of the regenerated plantlets using the DNeasy Plant Kit (QIAGEN, CA), following the manufacturer's instructions. The presence of the inserted genes was detected by PCR using different sets of primers; FP.hevP and RP1.hevP, RP2.hevP, or RP3.hevP primer combinations were employed to amplify the hevein promoter fragments, while HANF complete cDNA was amplified with HANF-For and HANF-Rev1. The plant selectable marker, *nptII* gene, was amplified using the FP.nptII (forward) and RP.nptII (reverse) primers.

Total RNA was extracted from newly expanded green leaf samples of selected transformed plants using the RNeasy Plant Mini Kit (QIAGEN, CA), and subjected to DNaseI (QIAGEN, CA) digestion to eliminate contaminating genomic DNA. RNA levels were estimated from ethidium bromide staining of ribosomal RNA separated on agarose gel. RT-PCR was performed using HANF-For and HANF-Rev1 primers according to the instructions in the OneStep RT-PCR Kit (QIAGEN, CA), while nested RT-PCR was performed using HANF-For and HANF-Rev2 (designed to bind further 3' to HANF-Rev1, in the reverse strand of HANF) primers to ensure a better re-amplification of the HANF generated in the initial RT-PCR. The amplified DNA fragment was ligated into pGEM-T vector and cloned into *E. coli* JM109 (Promega, CA). The recombinant pGEM-T vectors were harvested from the resulting white colonies and sequencing performed

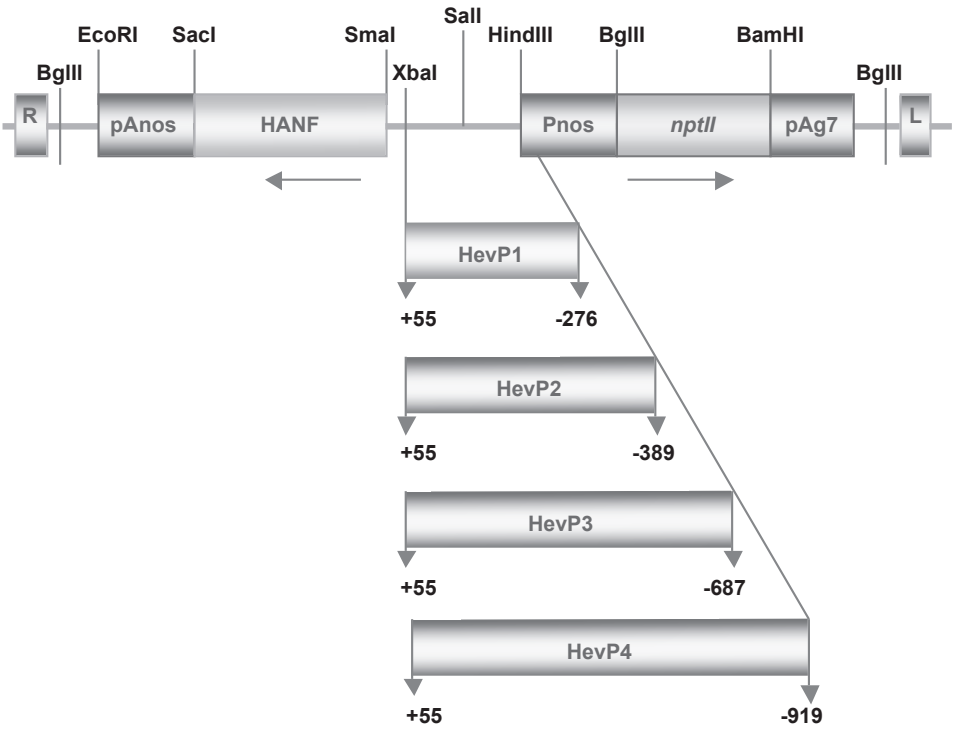

Figure 1. Schematic diagram of the pGPTV-Kan vectors with cloned HANF cDNA. The PCR-generated nested deletion fragments obtained from the hevein promoter region HevP and cloning sites are also shown. The 5' and 3' end points of the promoter sequences are numbered from the transcription start of the hevein gene. R indicates right T-DNA border, and L indicates left T-DNA border.

with T7 and SP6 primers that flank the insert. Sequence homology search was performed using the Blast[12] algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers used in this study are listed in *Table 1*.

Protein Analyses of *Hevea* Transformants

Newly expanded *Hevea* leaves (approximately 1 g) were washed in distilled water and blotted dry. Leaves were ground until finely powdered in liquid nitrogen. Total leaf protein from ground leaf tissue was extracted using the protocol described in Lebrun and Chevalier[13]. Quantitation of proteins present in the leaf extracts was performed using the Bradford assay[14]. About 100 µg of leaf protein was separated on 1 mm thick, 15% SDS-PAGE[15] using the BioRad® Mini-Protean II apparatus. Following electrophoresis the gels were either electroblotted to nitrocellulose[16] or stained with Coomassie Brilliant Blue R250 (Sigma, MO). Nitrocellulose membranes containing immobilised proteins were blocked with Tris-buffered saline containing 5% (w/v) non-fat milk powder. Human atrial natriuretic peptide C-terminus peptide, catalogue number A6916 (Sigma, MO) was the standard positive control used in SDS-PAGE. Proteins

TABLE 1. PRIMERS EMPLOYED IN PCR, RT-PCR AND NUCLEOTIDE SEQUENCING

Primer name	Primer sequence in 5' – 3' direction
ANF-for	TTT CCC GGG ATG AGC ACC TTC TCC ACC
ANF-rev	TTT GAG CTC TCA GTA CCG GAA GCT GTT
HANF-For	ATG AGC TCC TTC TCC ACC AC
HANF-Rev1	GTA CCG GAA GCT GTT ACA GC
HANF-Rev2	GC CCA GTC CGC TCT GGG CTC
FP.nptII	GAG GCT ATT CGG CTA TGA CTG
RP.nptII	ATC GGG AGC GGC GAT ACC GTA
FP.hevP	GG TCTAGA CCC ATT TCT TCC CAA TTC
RP1.hevP	GG AAGCTT CCT GGC CCT ATG CTC TAT
RP2.hevP	GG AAGCTT CGA GTT AAC CCT TGC GTT
RP3.hevP	GG AAGCTT GCC CTC TTG GTT GTT GCC
T7	GAG GCT ATT CGG CTA TGA CTG
SP6	ATC GGG AGC GGC GAT ACCGTA

transferred to nitrocellulose membranes were probed with goat polyclonal antibody (1:500) raised against human atrial natriuretic peptide C-terminus, catalogue number A5050 (Sigma, MO). Rabbit anti-goat IgG (1:1000) conjugated to alkaline phosphatase, catalogue number A4187 (Sigma, MO) was employed to label and visualise immunoreactive bands. The corresponding region on the Coomassie Blue stained gel was excised and MALDI TOF-TOF analysis was performed at the Proteomics Centre, National University of Singapore, Singapore.

RESULTS

Human Atrial Natriuretic Factor (HANF) Gene in Transformed Plants

Agrobacterium mediated genetic transformation of GL1 anther callus with HANF, under the control of four overlapping lengths of hevein promoter was performed. The kanamycin-resistant putative transformed callus underwent several rounds of sub-culturing for proliferation of callus. The presence of the HANF and *nptII* transgenes was established by PCR using DNA extracted from the primary callus. The bulk of the transformed callus (*circa* 3000) was sub-cultured and transferred into differentiation media to induce embryoids formation and subsequent plantlet regeneration. A total of 29 transformed plants were obtained. Eleven were HANF-HevP1 plants, seventeen HANF-HevP2 plants and one HANF-HevP3 plant. However, the HANF-HevP3 did not survive after transfer into polybag. The HANF-HevP4 callus on the other hand did not produce any transformants. The remaining 28 surviving plants are maintained in the netted plant house at the RRIM Research Station at Sungai Buloh. Bud grafting of the original transformed plants (T_0) is in progress as a preparation for transplanting in a confined field trial. PCR of leaf genomic DNA of HANF-HevP1 plants (original and the bud-grafted generations) with HANF-Rev1 and RP1.hevP primers amplified a *circa* 800 bp fragment that corresponds to the HANF-linked HevP1 insert. Similarly genomic DNA amplification of HANF-HevP2 plants with HANF-Rev1 and RP2.hevP gave a *circa* 900 bp amplicon that corresponds to the HANF-linked HevP2 insert (*Figure 2*). Nucleotide sequencing and Blast-N search confirmed the authenticity of the HANF-linked HevP1 and HevP2 fragments.

Human Atrial Natriuretic Factor (HANF) Transcript in Transformed Plants

RT-PCR performed on leaf total RNA, showed inconsistent amplification of HANF transcript from a random selection of original transformed plants and their bud-grafted generations (data not shown). To date, the detection of HANF transcripts was consistent only in one of the original transformant *i.e*. HANF-HevP2 plant number 179, and its vegetative generations. A representative RT-PCR was performed on selected transformed plants is shown in *Figure 3*. The HANF amplified fragment (*circa* 450 bp) was cloned into pGEM-T vector and subsequently had its nucleotide sequence determined. Blast-N search revealed an exact match of the generated nucleotide sequence to the HANF (IMAGE clone 306592), besides homology to other related entries in the databases.

Human Atrial Natriuretic Factor (HANF) Protein in Transformed Plants

When protein extracts from leaf tissue of the transformed plants were probed with the goat polyclonal anti-atrial natriuretic peptide, a distinct band was obtained at low molecular weight region (< 10k Da) in the transformed plant number 179. Very faint signals were discernible in the other transformed plants but absent from the non-transformed plant. The C-terminus atrial natriuretic partial peptide (A6916; Sigma, MO) employed as the standard protein may explain the slightly lower signal discerned as compared to the transformed plants in the Western-immunoblot (*Figure 4*). The corresponding protein band (plant number 179) was excised from a duplicate Coomassie Brilliant Blue stained gel, digested with trypsin and analysed with MALDI TOF-TOF. Data obtained from the mass spectra revealed three peptides that matched the C-terminus atrial natriuretic peptide of HANF in the tested leaf sample (*Figure 5*).

DISCUSSION

This paper reports on *Agrobacterium* mediated genetic transformation of *Hevea* cultivar GL1 with the cDNA encoding HANF. The HANF protein belongs to the natriuretic peptide family. Natriuretic peptides are implicated in the control of extracellular fluid volume and electrolyte homeostasis. HANF is synthesised as a precursor (*circa* 15 kDa), containing a signal peptide, which is processed to release a small peptide from the N-terminus (30 amino acid residues) with similarity to vasoactive peptide, cardiodilatin, and another peptide from the C-terminus (28 amino acid residues) with natriuretic activity[9] as shown in *Figure 5*. Genetic transformation with constructs bearing the four overlapping hevein promoter fragments, fused to the HANF gene in the pGPTV-Kan vector have generated 28 original transformed (T_0) plants *i.e.* eleven for HANF-HevP1 and seventeen for HANF-HevP2. The authenticity of all the transformed plants and their vegetative propagations was confirmed from the detection of HANF-linked hevein promoter inserts in genomic PCR analysis. RT-PCR screening on leaf total RNA revealed consistent amplification of HANF transcript in at least of one transformant (T_0 plant number 179) and its vegetative generations. The detection of HANF transcript reflects on the functionality of hevein promoter in leaf tissue. The functionality of hevein promoter has been demonstrated in the leaves[17] and latex[7] of transgenic *Hevea*. Moreover, MALDI TOF-TOF analysis revealed tryptic peptides that matched the mature C-terminus atrial natriuretic peptide of HANF precursor. Taken together these results confirm HANF expression in the transformed plant, and presumably the atrial natriuretic peptide is

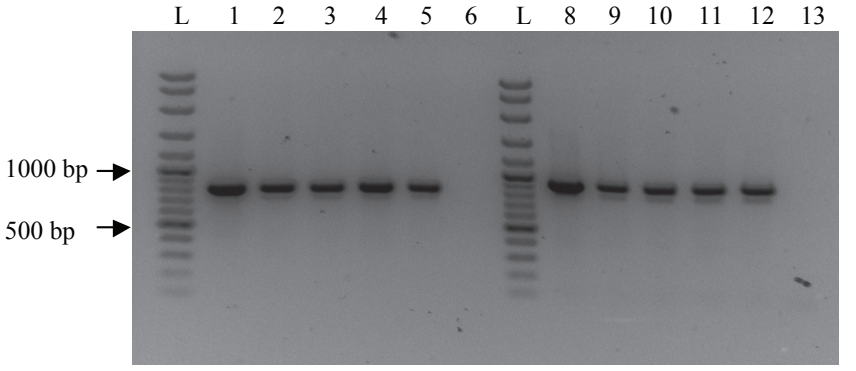

Figure 2. PCR amplification of hevein promoter-linked HANF from leaf genomic DNA of transformed plants. Amplifications were performed with reverse primer (HANF-Rev1) of HANF and reverse primers (RP1.hevP or RP2.hevP) of hevein promoter. L – 100 bp molecular ladder; lane 1, pGPTV-Kan-HANF-HevP1 (positive control); lanes 2 – 5, HANF-HevP1 plants (circa 800 bp); lanes 6 and 13, non-transformed plants (negative control); lane 8, pGPTV-Kan-HANF-HevP2 (positive control); lanes 9 – 12, HANF-HevP2 plants (circa 900 bp).

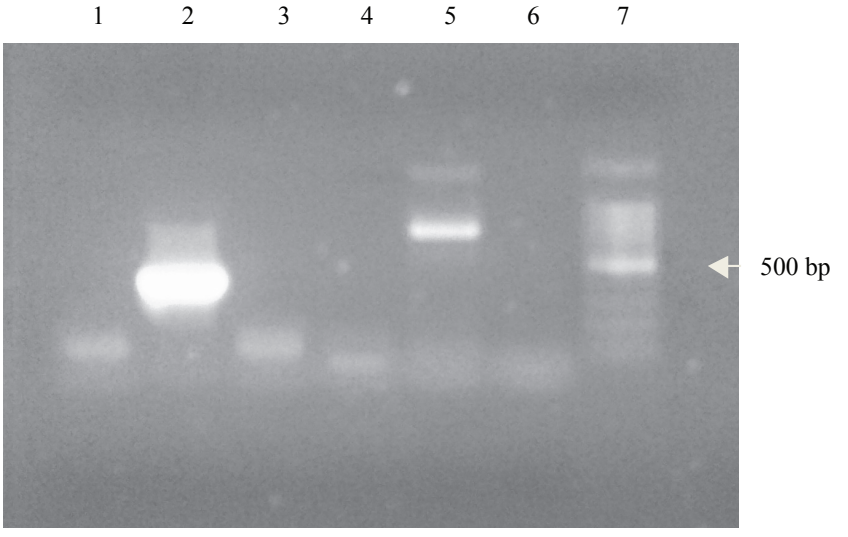

Figure 3. Nested RT-PCR showing amplification of HANF (circa 450 bp) of HANF-HevP2 transformant (plant number 179) in lane 2; no amplification at the expected size is discerned in the other transformed plants (lanes 1, 3, 4), the non-transformed plant (lane 5), water (lane 6) and 100 bp molecular ladder (lane 7).

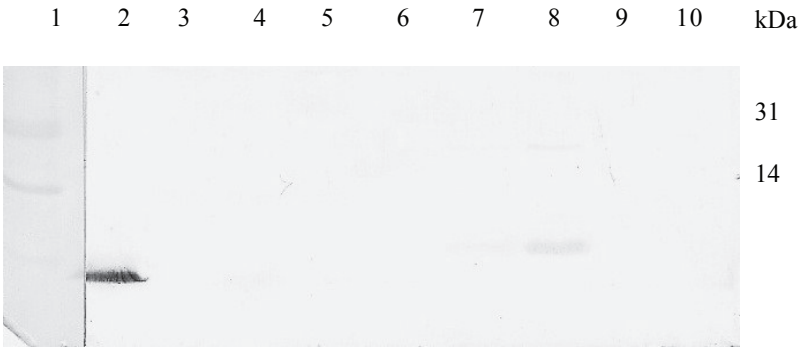

Figure 4. Western-immunoblot of SDS-PAGE separated leaf proteins reacted with goat polyclonal anti-human atrial natriuretic C-terminus peptide. Lane 1, Coomassie Blue stained molecular weight marker; lane 2, standard human atrial natriuretic C-terminus peptide; lanes 3 – 9, transformed plants; lane 10, non-transformed plant. The distinct band discerned in lane 8 (plant number 179) of the corresponding gel was excised and subjected to MALDI TOF-TOF analysis.

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      1                                                          60
HANF  MSSFSTTTVSFLLLLAFQLLGQTRANPMYNAVSNADLMDFKNLLDHLEEKMPLEDEVVPP

      61                                                         120
HANF  QVLSEPNEEAGAALSPLPEVPPWTGEVSPAQRDGGALGRGPWDSSDRSALLKSKLRALLT

      121                              153
HANF  APRSLRRSSCFGGRMDRIGAQSGLGCNSFRYRR
  p1                IDRIGAQSGLGCN
  p2          SSCFGGR
  p3          SSCFGGRIDR
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Figure 5. Alignment of the MALDI TOF-TOF generated peptides (p1, p2 and p3) of transformed plant (number 179) with the complete amino acid sequence of human atrial natriuretic factor. The mature peptide regions of HANF are highlighted: residues 26-55 correspond to the N-terminus cardiodilatin peptide, while residues 124-151 correspond to the C-terminus atrial natriuretic peptide.

derived from post-translation cleavage of the HANF precursor. It remains to be seen if the mature cardiodilatin peptide in the N-terminus of HANF is expressed in the transformed plants when the specific antibody is available. Further investigations shall reveal HANF expression particularly in the latex cytosol, both in T_0 and the vegetative generations of the transformants in a confined field trial.

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