CHAPTER 4
MATERIALS AND METHODS

4.1. PLANT MATERIAL

For the whole research work, the healthy *Stevia rebaudiana* plants were maintained as a garden at SCMS institute of Bioscience and Biotechnology Research and development (SIBB R&D), Cochin. For the biochemical studies, the fresh mature leaves were collected from healthy *Stevia rebaudiana* plants prior to flowering. For the molecular studies the *Stevia* plants grown in pots maintained in the green house were used. Fresh, healthy and infection free tender leaves, mature leaves, stem and flowers of 3-4 months old plants were selected for real time expression studies.

4.2. QUANTIFICATION OF STEVIOL

4.2.1. Extraction from plant material

Steviol was quantified by the method of Minne *et al.*, (2004). 100 mg of freeze dried powdered leaves of *Stevia rebaudiana* plant was prepared. The powder was homogenized with 0.5 ml of acetone and the solvent was evaporated. The residue was three times extracted with 0.5 ml of KOH at 4°C in methanol with continuous vortexing in a micro centrifuge tube. After extraction, 5 ml of water was added to the combined extracts and the lipids were removed by three extractions with 5 ml of diethylether. The water phase was
then acidified to pH of 6 by the addition of 6 N acetic acid for preventing the conversion of steviol to isosteviol. The steviol was then extracted with equal volume of peroxide free diethyl ether. The combined ether fractions were evaporated to dryness and the water free steviol source was collected and dissolved in acetone.

4.2.2. Estimation of steviol

Steviol was measured spectrophotometrically at an absorbance of 210 nm against blank devoid of steviol extract.

4.3. QUANTIFICATION OF STEVIOSIDE

4.3.1 Extraction from the plant material

Stevioside was isolated by following the method of Brandle et al., (1998). For the isolation of stevioside, the mature leaves of Stevia plants were chopped and boiled in water for 2 hrs and homogenized in a mortar with a pestle. The homogenate was filtered through a double layered fine cheese cloth. The extract was centrifuged at 10,000 rpm for 10 min. The supernatant was collected and made up to known volume and was used as the stevioside source.

4.3.2. Estimation of stevioside

50 µl extract was taken from the stevioside source and made up to 25 ml using distilled water. The absorbance was measured at 200 nm against the blank devoid of stevioside extract.
4.3.3 High performance liquid chromatography of stevioside and rebaudioside

Qualitative fractionation of stevioside and rebaudioside by high performance liquid chromatography was carried out by the method of FAO JECFA (2008). Stevioside and rebaudioside extract from the mature leaves of Stevia were used for the study against the purified stevioside and rebaudioside as standard (Sigma ALDRICH).

4.3.3.1. Preparation of sample

1 g leaf tissue was finely chopped and refluxed in 80% methanol for 30 mins. After cooling, the tissue was homogenized by grinding in a mortar with a pestle. The homogenate was filtered through a cheese cloth and evaporated. The remaining residue was dissolved in mobile phase and used for HPLC analysis.

4.3.3.2. Mobile phase preparation

HPLC grade acetonitrile was used for the mobile phase preparation. Acetonitrile (800 ml) was mixed thoroughly with deionized water (200 ml) and the pH was adjusted to 3.0 with acetic acid. The mobile phase obtained was filtered through 0.22 μm nylon membrane filter (Millipore) for degasification.

4.3.3.3. HPLC procedure

HPLC analysis of stevioside and rebaudioside was done by a HPLC system (Water associates) equipped with 7725 Rheodyne injector and waters 510 HPLC pump, 486 Tunable absorbance detector and Millenium 2070 software data module. The sample was injected to silica based aminopropyl
bonded sorbent column (4.6 x150 mm) at room temperature. The mobile phase was acetonitrile: water (pH 3.0, adjusted with acetic acid) (80:20, v/v) and flow rate was adjusted to 1.0 ml/min at 210 nm wavelength for 20 minutes (FAO JECFA .2008). 20μl of sample was injected and quantification at 210 nm was recorded. As standards, stevioside (1 mg/ml) and Rebaudioside(1 mg/ml) extract prepared from stevioside and rebaudioside standards (Sigma ALDRICH) were injected into the column separately. Stevioside and rebaudioside concentrations of the samples were identified by comparing with the retention time of the standards. Areas of the peaks were taken for the quantification. Concentration of the standards and area of the peak were taken as the standard parameters.

4.4. ENZYME ASSAY OF URIDINE DIPHOSPHATE GLUCOSYL TRANSFERASE.

Enzyme extract was prepared in 0.1 M-sodium phosphate buffer, pH7.5, and used immediately after preparation. Enzyme activity was determined an assay which contained of 3 ml containing 0.167 mM-p-nitrophenol, 0.833 mM -UDP-glucose, 16.7 mM- MgCl2, 66 mM-sodium phosphate buffer, pH 7.5, and enzyme extract equivalent to 1 g fresh wt. of leaves (50 mg of protein). To the reaction 100 μl 1 M Triton X-100 was added (Madina, et. al., 2007). The reaction was started by the addition of UDP glucose and incubation was carried out at 35°C. At timed intervals of 30 min, 0.6 ml of assay mixture was added into tubes containing 2.4 ml of 0.1M TCA to stop the reaction. The acid-insoluble material was removed by centrifugation (5000 g for 10 min), and 0.06 ml of 12 M KOH was added to the supernatant. The decrease in absorbance at 400 nm in the supernatant was determined in a spectrophotometer.

4.5. RNA ISOLATION FROM STEVIA LEAF

Total RNA from the plant tissues was isolated by Guanidium Iso Thiocyanate (GTC) method with slight modifications (Chomczynski and Sacchi, 1987). Fresh leaf sample was collected from healthy plants of S. rebaudiana
from the experimental garden of the institute. Approximately 100 mg of tissue was homogenized in a pre-chilled mortar, using liquid nitrogen, into a fine powder 500 µl of solution D (containing 4M Guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosine and 0.1 M β-Mercaptoethanol) was added and the fine powder was made into slurry. The slurry was then transferred to a clean, sterile eppendorf tube (1.5 ml). 100 µl sodium acetate (2M, pH 4.0), 500 µl water saturated phenol (pH 4.3, pH was adjusted with citric acid), 200 µl chloroform: isoamylalcohol (24:1) were added and vortexed for 5 min between each addition. The resulting mixture was then incubated on ice for 15 min and centrifuged at 10000 g for 10 min at 4°C. The upper aqueous phase was carefully collected in a fresh eppendorf tube. Two volumes of 95% ethanol were added, gently inverted the tube for 3 min and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was decanted and the pellet was treated with 70 % ethanol for washing. The supernatant obtained after centrifuging it in 10000 g was decanted out and the pellet was air dried for removing the traces of ethanol present in the pellet and was dissolved in minimum volume of RNA free sterile water and was stored at -80°C until use. The purity of RNA was checked by formaldehyde gel electrophoresis and the concentration of RNA was quantified at 260 nm.

**4.6. DESIGNING OF PRIMERS AND REVERSE TRANSCRIPTION**

**4.6.1 Primer designing**

The gene specific primers for the amplification of UGT genes were designed from the accession numbers AY345978, AY345982 and AY345974 using the software Vector NTI (Invitrogen), Life technologies. Appropriate primers were designed by maintaining the quality.
4.6.2 Reverse transcription

The first strand synthesis was done in a total volume of 25µl containing 4 µg of RNA, 2.5 µl of 10X buffer, 15 U RNase inhibitor, 1 mM dNTP’s, oligo dT (100 ng) and 250 U of MmuLV reverse transcriptase. In the initial step, the RNA was incubated with reverse primer for 10 min at 65°C and snap chilled in ice for 2 min. The other reaction components were added and incubated at 37°C for one hour followed by 70°C for 10 min and then held at 4°C.

4.7. AMPLIFICATION OF UGT GENE

The PCR reaction was carried out in 25 µl reaction mixture containing 2 µl of cDNA template, 10mM dNTP, 25 mM MgCl₂, 20 pmol gene specific primers and 1 units of Taq DNA polymerase. The reaction condition was performed with an initial denaturation for 4 min at 95°C , 35 cycles of 30 sec at 95°C, 40 sec at 56°C and 2 min72°C, followed by final elongation of 10 min at 72°C. The PCR product was analyzed by 1.0% agarose gel electrophoresis.

4.8. CLONING OF UGT GENES TO PTZ57R/T VECTOR

4.8.1 Gel elution of PCR product

The PCR amplified 1450 bp UGT gene of interest was eluted from the gel using Gel DNA recovery kit (Genei, Bangalore). The amplified product was run on 1.0% agarose gel to separate the DNA fragment of specified band size. The desired DNA amplicon were excised from the gel and placed in a pre weighed micro centrifuge tube. The net weight of gel piece was determined and 2.5 volumes of sodium iodide solution was added to it and incubated at 45-55°C for 5 min for solubilizing the gel. 15 µl of homogenous glass solution mixture was added to the tube, vortexed and incubated at room temperature for 5 min. The sample was centrifuged at 12,000 rpm for 30 sec and the supernatant discarded. The pellet was washed with wash buffer 2-3 times and centrifuged at
12,000 rpm for 30 sec. The pellet was kept at 55°C for 10 min in order to remove any traces of wash buffer. The pellet was resuspended in 20 μl of water and incubated at 45-55°C for 5 min. It was centrifuged at 12,000 rpm for 30 sec and the supernatant collected in a fresh tube. 10 μl of water was added to the pellet and resuspended. This was incubated at 55°C for 5 min. A final spin at 12,000 rpm was done for 30 sec and the supernatant collected in a fresh tube. The eluted PCR product was checked in 1.0% agarose gel and it was stored at -20°C.

4.8.2 Ligation of the product into pTZ57R/T vector:

The gel eluted UGT genes and the linearized TA cloning vector pTZ57R/T vector were used for ligation reaction. 20 μl ligation reaction contained 110 ng pTZ57R/T vector (Fig.4.1), 5 μl of 4 X buffer, 330 ng eluted PCR products of UGT genes and 5 U of T4 DNA ligase. The ligation mixture was mixed well and incubated overnight at 22°C and directly used for transformation.

4.8.3 Transformation of Recombinant Plasmids

4.8.3.1 Competent cell preparation of E.coli JM109

Single colony from fresh plate of E.coliJM109 was inoculated in 5 ml sterile LB medium and incubated at 37°C overnight with appropriate shaking. 1 ml of overnight culture was then transferred to 50 ml sterile LB and kept at shaking at 37°C until OD₆₀₀ reaches 0.320. The culture was then immediately kept on ice for 15 minutes. After incubation the cultures were centrifuged at 1600 g for 10 minutes 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml 100 mM sterile calcium chloride, followed by centrifugation at 1100 g at 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 750 μl calcium chloride and 250 μl glycerol and stored at -80°C in 50 μl aliquots.
Fig. 4.1 Vector map of TA cloning vector pTZ57R/T
4.8.3.2 Transformation of clone into competent cells

10 µl of ligation mixture was used for the transformation of JM109 competent cells by heat shock method at 42°C for 2 min. The selection of transformants were done by blue white screening on LB agar plates containing 50 µg/ml of ampicillin, 0.1 mM of IPTG and 40 µg/ml of X-Gal. The white colonies representing recombinant plasmids were selected for further studies.

4.8.4 Confirmation of the clone using restriction digestion

The cloned plasmids were confirmed by doing restriction digestion with EcoR1 and BamH1 for UGT1, EcoR1 for UGT2 and Sac I for UGT3 genes. The restriction digestions were performed in a final volume of 20 µl containing, 2 µl 10 X buffer, 10 units of EcoR1 (1 µl) & 10 units of BamH1 (1 µl) for UGT1, 10 units (1 µl) of EcoRI restriction enzyme for UGT2 and 10 units (1 µl) sac I restriction enzyme for UGT3, 2 µg of plasmid DNA and remaining autoclaved water in 1.5ml sterile eppendorf tubes. The contents were mixed well and incubated at 37°C for 2 hours in a thermomixer. After incubation, 5 µl of the samples were loaded on 1.5% agarose gel along with appropriate DNA molecular weight marker and was documented.

4.8.5 Sequencing and Sequence Analysis

The UGT- pTZ57R/T plasmid was subjected to automated sequencing (Xcelris, Hyderabad). The sequence of the gene was analyzed using Bioedit programme (Hall, 1999). The restriction map was created based on the sequence analysis. The sequence homology of the gene was done by blast search.
4.9. STRUCTURE PREDICTION OF UGT GENES

4.9.1 Analysis and comparison of amino acid sequences

Vector NTI software employed for comparative analysis, exercises a progressive algorithm based method that not only provides better average accuracy but better speed and hence was employed for multiple sequence alignment. The translated sequences were then again compared to check if these variations cause substitution of one amino acid for another in the protein (missense mutation). Physicochemical analysis of the sequences namely molecular weight, theoretical pI, amino acid composition, instability index, aliphatic index and grand average of hydropathicity (GRAVY) were performed using PROTPARAM tool.

4.9.2 Structure prediction and analysis

Translated DNA sequence of UGT’s were modeled using Phyre software which implements comparative protein structure modeling by spatial restraints to construct protein models (Kelley and Sternberg, 2009). All UGT protein (UGT1, UGT2 & UGT3) variants were modeled using structure of ‘UGT85H2 Medicago truncatula’ (PDB ID: 2PQ6) obtained from protein data bank. To gain more insight in the locations of the amino acids, the domains were predicted and analyzed using PDBsum from Procheck (Laskowski, 2009) Procheck verifies the stereo chemical quality of a protein structure and provides an at-a-glance overview of every macromolecular structure deposited in the Protein Data Bank. PyMOL program was employed for interactive visualization and analysis of molecular structures (Delano, 2002). Structural superposition was performed between the UGT 1 & UGT74 G1 protein, UGT2 & UGT85C2 protein and UGT 3 & UGT76G1 protein sequences using a combination of
Match Maker and Match-Align subroutine of Chimera (Pettersen et al., 2004). A novel score incorporating both secondary structure and residue type is used to align the sequences in Match Maker while Match-Align constructs sequence alignments from pre-existing super positions of structures. These tools when used together enhance the understanding of sequence information in the context of structure and vice versa.

4.9.3 Binding pocket exploration

Surface topography of proteins was computed using CASTp server (Dundas et al., 2006). The weighted Delaunay triangulation and the alpha complex for shape measurements method is utilized by CASTp server that compute identification and measurements of surface accessible pockets as well as interior inaccessible cavities, for proteins and other molecules. This tool measures analytically the area and volume of each pocket and cavity, both in solvent accessible surface and molecular surface (Anisha et al., 2012).

4.10. REAL TIME PCR

For the relative quantification of the gene expression, RNA was extracted from the tender and mature leaves, stem and roots. An amount of 4.0 mg of total RNA was reverse transcribed with 5U of AMV reverse transcriptase in a total volume of 25 ml reaction containing 100 ng of Poly T primer (Fermentas, USA), 20 U RNase inhibitor and 1 mM dNTPs. RT quantitative PCR was conducted in Eppendorf Master cycler ep realplex using the PCR cycle conditions 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 15 s 60 °C and 45 s at 72 °C and followed by melting curve analysis. Each 20 ml reaction contains 5 ml cDNA (10 times diluted), 1X SYBR green master mix (Eppendorf, Germany) and 3 pmoles of reverse and forward primers of both internal control (Stevia actin) and gene of interest (UGT3) were used. Relative standard curves generated with serial dilutions (10x) of previously prepared cDNAs were used
for efficiency curve determination. All expression data analyses were performed after comparative quantification of amplified product using the $2^{-\Delta\Delta C_t}$ method as previously described (Livak et.al, 2001, Bustin et.al, 2009).

4.11. PROKARYOTIC EXPRESSION OF UGT3 GENE

UGT3 full gene was subcloned into prokaryotic expression vector pET32c and clone was transformed into *E.coli* BL21DE3pLys cells. For prokaryotic expression, a single colony from the transformed plate containing pET32c-UGT3 clone was inoculated into 5 ml sterile LB broth containing ampicillin (50 µg/µl). The culture was grown at 37°C until the OD$_{600}$ reached 0.6-1. The culture was stored in refrigerator for overnight; 2 ml culture was centrifuged at 10,000 rpm for 2 min at 4°C. The pellet was resuspended in 2 ml fresh LB broth containing ampicillin and was used to inoculate 50 ml of LB ampicillin medium. The culture was grown at 37°C to an optical density (OD$_{600}$) reached 0.4-1. The protein expression was induced by adding IPTG at a final concentration of 1 mM for 3 hrs shaking at 37°C. 1 ml each bacterial cultures were drawn at a time interval of 0 hr, 1 hr, 2 hr and 3 hrs. The expression profile was analyzed by SDS-PAGE.

4.12. PURIFICATION OF RECOMBINANT UGT3 PROTEIN

For purifying the recombinant UGT3 protein, the 50 ml culture after 3 hrs of induction was centrifuged at 12000 g for 10 min at 4°C and the pellet was resuspended in 1 ml ice-cold PBS (pH 7.4). The cell suspension was centrifuged and the PBS wash was repeated twice. The cell pellet was finally resuspended in 1 ml PBS and 5 µl aprotinin (1 mg/ml) and 10 µl of lysozyme (10 mg/ml) were added. Freeze-thaw lysis was done in liquid nitrogen (20 seconds) and kept in warm water (37°C) for 1 minute. The above cell lysis step was repeated 10 times. DNase (1 mg/ml) and RNase (1 mg/ml) was added to lysate for reducing the viscosity of cells. Total cell lysate was incubated at 4°C for 10 min with shaking and clarified by centrifugation at 10,000 g for 30
minutes at 4°C. Both supernatant and pellet were submitted to 12% SDS-PAGE for the identification of soluble fraction (Simionatto, et. al., 2010).

Cell lysis was done by repeated freeze-thawing in liquid nitrogen (20 sec) and at 37°C (1 min). The freeze-thaw was repeated 10 times. Then 10 μl of DNase (1 mg/ml) and Rnase A (10 mg/ml) was added to the lysate. The lysate was incubated at 4°C for 10 min with shaking. After the incubation the lysate was centrifuged at 12,000 g for 30 min at 4°C. The inclusion body pellet was resuspended in solubilizing buffer containing 200 mM NaH₂PO₄, 500 mM NaCl, 5 mM Imidazole and 8 M urea pH 8.0. Cell lysate was incubated at 4°C. for 60 minute and soluble protein was recovered by centrifugation at 14,000 g for 60 minutes. The recombinant protein was purified by affinity chromatography using Nickel coated IMAC Hypercel column (PALL) according to the manufacturer’s instructions. The protein was eluted using 200 mM Imidazole. The eluted protein was dialyzed against PBS containing decreasing concentrations of urea. The concentration of protein was estimated by Bradford assay.

4.13.1 Development of Polyclonal antibody against UGT3 protein

The recombinant UGT3 produced by E.coli expression system was used as a suitable antigen for elicitation of polyclonal antibodies. The UGT3 was found to be insoluble in nature. So the IPTG induced bacterial cells are directly denatured in 1X SDS-loading buffer and loaded on 12% SDS-PAGE. The 34 KDa protein is excised out of gel and fragmented using homogenizer in 1X PBS. The gel slice containing the recombinant UGT3 protein was homogenized in 1X PBS and combined with 0.625 ml of the Freunds complete adjuvant. The antigen and adjuvant were mixed thoroughly to form a stable emulsion which was injected subcutaneously beneath the skin at an area around the shoulder of the New Zealand white rabbit maintained in the animal house.
Pre-immunization bleeding of the animal: Blood was collected from the central ear artery with a 19 gauge needle and allowed to clot and retract at 37°C overnight. The clotted blood was then refrigerated for 24 h before the serum is decanted and clarified by centrifugation at 5000 rpm for 20 min.

Schedule for immunization

Day 0 – Pre immune bleed, first immunization (50-100 μg antigen, CFA)
Day 14 – Second booster doze (50-100 μg antigen, IFA)
Day 22 – Test bleed
Day 35 - Third antigen booster
Day 42 – blood collection

Serum separation from blood

The collected blood was incubated at 37°C for 1 h. It was kept for overnight incubation at 4°C and centrifuged at 2000 rpm for 10 min for collecting serum. To the collected serum ammonium sulphate was added at a final concentration of 50% for the precipitation of antibody and dialysed against PBS to remove the salt. The salt precipitated antibody was subjected to purification using protein A agarose column. The column equilibrated by applying 10 bed volume of 1X equilibration buffer. The serum samples were applied to the equilibrated column. About 2-4 ml of the sample was added onto 2 ml column. Then the column was washed with 25 bed volume of 1X equilibration buffer. Elution of antibody was done using 1X elution buffer and the antibody fractions of 1 ml each were collected in separate tubes. The absorbances of all the fractions were measured at 280 nm. Antibody fractions showing absorbance above 0.3 were pooled out.
Total IgG was estimated as:

\[
\text{Absorbance} \times \text{Dilution factor} = \text{Equation 1}
\]

1.4

Where 1.4 is the extinction coefficient of IgG.

The purified antibody was then analyzed by 12% SDS-PAGE analysis.

4.13.2 Western blot analysis

Protein sample which was resolved by SDS-PAGE analysis were electrophoretically transferred onto a nitrocellulose membrane for Western blot analysis (Cho et al., 2008). The run was carried out in blotting buffer (48 mM Tris, 39 mM glycine, 20% methanol and 0.04% SDS) for 2 h at 50 volt. The nitrocellulose membrane was subjected to overnight blocking in TBS containing 5% BSA at 4°C. The membrane was then reacted with 1:1000 dilution of primary antiUGTTigG in TBS containing 2% BSA for 2 h at 37°C. Subsequent to this the membrane was washed with the wash buffer, TBS containing 0.05% Tween 20 and incubated with horseradish Peroxidase (HRP) conjugated with goat antirabbit IgG antibodies (Bangalore, Genei) at a dilution of 1:5000 in conjugated buffer containing 1.5% BSA for 1 h at 37°C. Finally the blot was stained with precipitated 3,3’,5,5’-tetramethyl-benzidine solution containing H₂O₂ (Bangalore, Genei) till the colour developed. At the end, the reaction was terminated by treating with water. The gel pattern was recorded immediately (Yati et al., 2011).
4.13. EUKARYOTIC EXPRESSION OF UGT3 IN CELL SUSPENSION CULTURES IN *NICOTIANA TABACCUM*

4.13.1 Competent cell preparation of *Agrobacterium tumefaciens* EHA 105 and transformation

One colony of *A. tumefaciens* EHA 105 was inoculated in 25 ml sterile LB broth containing 20 µg/ml rifampicin. The culture was allowed to grow at 28°C, 220 rpm till OD<sub>600</sub> became 0.8, the culture was transferred to a tube and incubated at 4°C for 5 minutes and centrifuged at 5000 rpm for 5 minutes at 4°C. The pellet was resuspended in 1 ml of chilled 20 mM sterile calcium chloride. To the 100 µl of above suspension, 1 µg of the pORECaMV-UGT3 plasmid was added. The aliquots were freeze-dried in liquid nitrogen followed by thawing at 37°C for 20 minutes. 1 ml of sterile LB broth containing rifampicin (50 µg/ml) was added to the tube and incubated at 28°C for 1 hour at 220 rpm. Kanamycin (1 µg/ml) was added to the tube. The tubes were incubated at 28°C for 2 hours at 220 rpm. 100 µl of the above culture was spread on an LB agar plate containing kanamycin (50 µg/ml) and rifamicin (20 µg/ml) and incubated at 28°C.

4.13.2 Cocultivation of *Agrobacterium* with *Nicotiana tobacum* suspension culture

Suspension cultures were established from 3 weeks old callus of *Nicotiana tobacum* in a 100 ml MS medium containing 250 mg/l 2-4D. The cultures were sub cultured every 15 days into fresh medium. To the 50 ml suspension culture, 500 µl of agrobacterium culture containing 50 mg/l kanamycin was inoculated followed by cocultivation in dark condition for 2 days. Cocultivated cells were centrifuged at 1000 rpm for 5 min at 25°C. The cell pellet was transferred into fresh MS medium (Murashige and Skoog 1962) followed by centrifugation. The above procedure was repeated for 4 times for
washing the cells to remove agrobacterium from the culture. After washing, co-
cultivated tobacco cells were cultured on MS medium containing 150 mg/l
kanamycin, 250 mg/l cefotaxim and 0.25 mg/l 2-4-D and cultivated in dark
condition at 25°C.

4.13.3 DNA isolation from suspension culture

Total DNA from suspension culture was extracted by modifying
the CTAB method of Roger and Bendich (1988) (Tchorbadjieva and Ivelin,
2004). The tissue samples were ground in liquid nitrogen to a fine powder using
a motor and pestle and transferred to a microcentrifuge tube. A 350 µl of 2X
CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl (pH-8.0), 1.4 M NaCl,
1% PVP preheated to 65°C) was added several times very gently to disperse
powder into solution. The tube was then incubated at 65°C for 5 minutes.700 µl
of chloroform/ isoamylalcohol (24:1) was added, vortexed for few seconds and
centrifuged 10,000 rpm for 1 minute. The step of deproteinization with
chloroform/ isoamylalcohol (24:1) was repeated twice.1ml of absolute ethanol
was added, mixed gently and centrifuged for 1minute in order to precipitate
DNA. The pellet was washed with 70% ethanol and centrifuged for 1minute. The
above step was repeated twice and the pellet was dried at room temperature. The
pellet was dissolved in 30 µl of autoclaved water and incubated at 65°C for 10
minutes to dissolve genomic DNA.

4.13.4 Analysis of UGT3 protein contents in transformed Tobacco culture
cell lines

For analysis of UGT3 content, the transformed Nicotiana cells
were ground in liquid nitrogen and the proteins were extracted with 0.1 M
phosphate buffer (pH 7.0) containing 10 µg/l aprotinin and centrifuged at 12000
g for 15 minutes at 4°C. To the supernatant 10% TCA was added and centrifuged
at 10000 rpm for 10 minutes at 4°C (Mitra and Zhang, 1994). The pellet was resuspended in 0.1 N NaOH and loaded in 12% SDS-PAGE.

4.14. ASSAY OF rUGT3 ENZYME

The rUGT3r expressed by both prokaryotic and eukaryotic system was subjected to the enzyme assay reaction for determining the functionality of the protein. The glycosylation of UGT3 was analyzed by HPLC. The substrate stevioside (0.5 mM) was incubated with 500 mg recombinant UGT3 for 1 h at 30°C in reaction mixture containing 1 mM UDP Glucose, 100 mM Tris HCl (pH 8.0), 5 mM MgCl2, 1 mM KCl, 0.1 U/ml calf intestine phosphatase (Shibata et al., 1995). After the incubation period, the reaction mix was centrifuged at 10,000 rpm for collecting the aqueous phase. The control reaction was done with water instead of enzyme source. The sample was injected to silica based aminopropyl bonded sorbent column (4.6 x150 mm) at room temperature. The mobile phase was acetonitrile: water (pH 3.0, adjusted with acetic acid) (80:20, v/v) and flow rate was adjusted to 1.0 ml/min at 210 nm wavelength.