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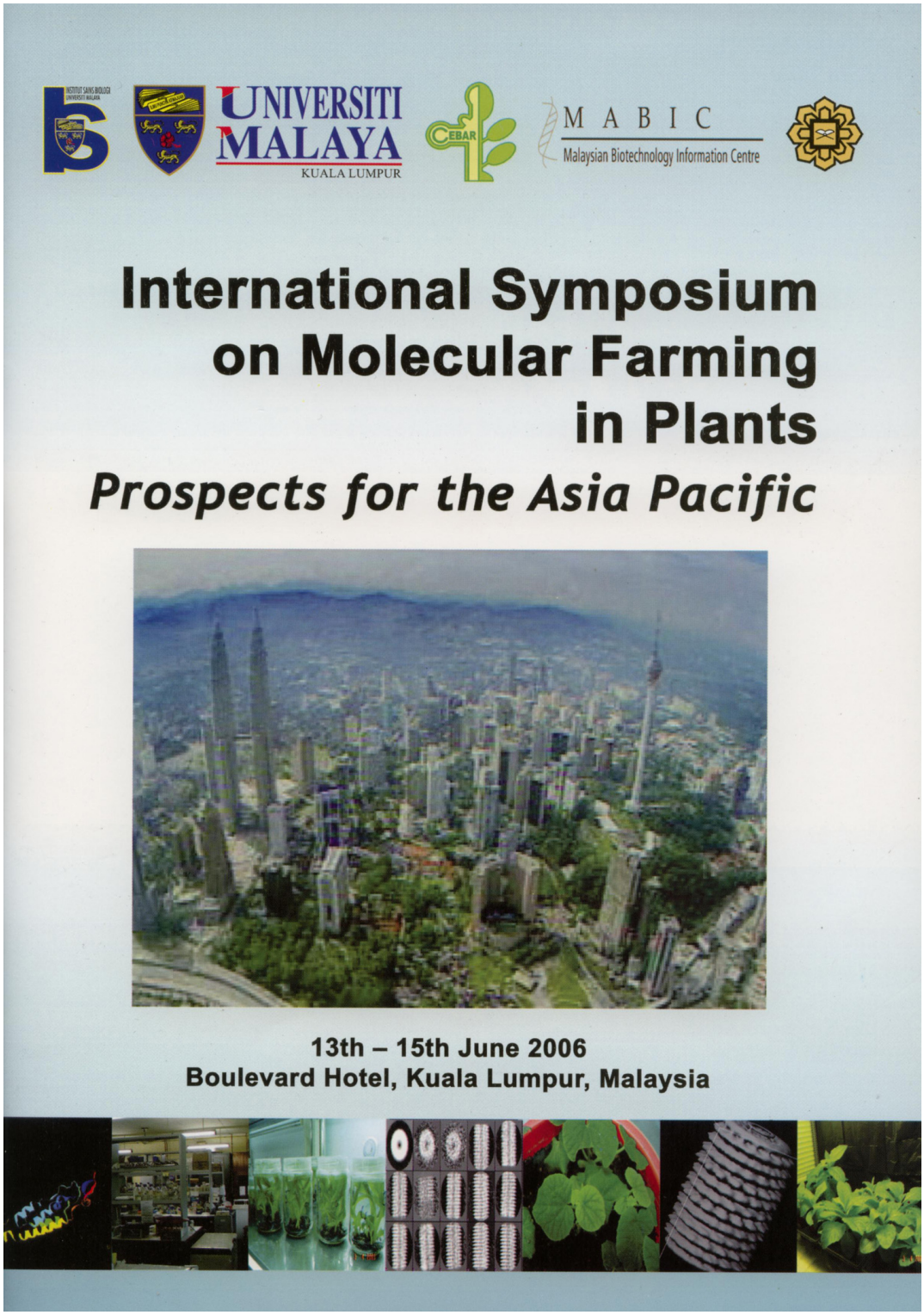
Abstracts



Expression of Human Immunodeficiency Virus and Human Papillomavirus Antigens in Plants as Candidate Human Vaccines

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There is an urgent need for inexpensive vaccines for the prevention of HIV-related diseases and high-risk papillomavirus-related cervical cancer in southern Africa, where HIV infections are rampant, where there is the highest rate of cervical cancer in the world, and where the bulk of the population have no hope of affording standard vaccines currently under development. We have accordingly explored the potential of a variety of expression techniques in a variety of plant types for the high-level expression of virus-like particles (VLPs) as vaccine antigens for HIV-1 subtype C and HPV types 11 and 16.

We have used the HIV-1 subtype C Du151 isolate gag gene in its native form as well as resynthesised versions with codon optimisation for human and *Nicotiana spp* expression. Expression of these genes was tested in *N. benthamiana* via a tobacco mosaic virus-derived (rTMV) vector, in transgenic *N. tabacum*, and in both plant types via *Agrobacterium tumefaciens* infiltration-mediated transient expression with a variety of expression plasmids. We also tested the expression of the similarly-optimised and native HIV-1C p24 capsid protein via TMV and via agroinfiltration in *N. benthamiana*, and of "plantised" p24 in transgenic tobacco: this was to explore the potential of making the protein as a cheap reagent for diagnostic purposes.

We have used native full-length and truncated forms of HPV types 11 and 16 L1 genes to make transgenic Arabidopsis thaliana (HPV-11 only) and tobacco and for expression in N benthamiana via rTMV, and have also used native HPV-11 L1 and plantised and humanised HPV-16 L1 genes for agroinfiltration and transgenic expression in N benthamiana and tobacco. We have investigated the effect of different intracellular localisation or targeting of vaccine proteins by means of a novel set of expression vectors. We have also done a proof of concept study for plant production of a papillomavirus vaccine, by testing the efficacy of plant-produced Cottontail rabbit papillomavirus (CRPV) L1 protein in a rabbit model.

We succeeded in getting expression of nearly all genes in all systems, with assembly of recognisable VLPs for both HIV and HPV. There was a marked difference in efficiency of expression for different genes, however: for example, plantised gag was expressed most efficiently of all gag versions tested in transient expression systems, while it was impossible to regenerate gag+ transgenic plants; native HPV-11 could only be efficiently expressed as a transgene if truncated so as to remove nuclear localization signals (NLS-), and then expressed in all systems at levels equivalent to the HPV -16 L1 humanised gene, which was far better expressed than native or plantised forms. P24 protein was best produced via rTMV, as the plantised version - however, rTMV was not as suitable for bigger HIV or HPV genes. Transgenic Arabidopsis was very efficient at producing HPV-11 L1 NLS-compared to tobacco. Agroinfiltration proved to be a very useful means of producing humanised HPV-16 L1. HPV-16 L1 was produced at highest levels when targeted to chloroplasts.

Various extraction protocols have been explored for their suitability for concentrating and purifying vaccine antigens, and this will be summarised. In preliminary results it was shown to be feasible to immunise mice and rabbits parenterally with concentrated and partially purified HPV L1 from transgenic or transiently-expressing plants: these studies and oral delivery studies on plant-derived antigens are ongoing, and will be described. The success of the CRPV test model in rabbits will also be detailed.

A Novel Expression Platform for the Production of High Value Proteins in Tobacco

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The production of novel products in plants has been an area of considerable activity for the past decade with particular emphasis on the production of plant made pharmaceuticals and vaccines. Numerous plant systems (tobacco, corn, lucerne, safflower, lemna amongst others) and transgene expression technologies (nuclear and chloroplast integration; conventional expression cassettes, inducible expression and oil body targeted protein fusions) have been reported. We have selected tobacco as the most appropriate plant host for the Australian "environment": it is easily genetically modified, it is a non-food crop, it is self fertile with very low rates of out-crossing, its production has been and continues to be highly regulated in Australia, the Australian tobacco industry has severely contracted in recent years and is committed to developing new tobacco based products and production in tobacco will probably experience fewer regulatory hurdles than food or feed crop species. We have developed a novel transgene expression technology termed INPACT for **IN P**lant **ACT**ivation. This expression system is based on the rolling

circle replication strategy utilised by ssDNA plant viruses, members of the Geminiviridae and the Nanoviridae and involves both activation and amplification of the transgene. This activation and amplification is directed by a Rep protein. The technology has specific application for the expression of "difficult" proteins, proteins that are either generally expressed at low level or inhibit plant growth or are toxic as the transgene is "split", with no expression in the absence of activation. Initial development of the technology has involved expression of the maker proteins, GUS and GFP, and activation by induction of the expression of the Rep using inducible promoters such as wound or ethanol inducible promoters. We have now applied the technology to the production of a high value medical protein, vitronectin. Vitronectin is a blood protein widely used in tissue culture media as an enhanced of cell attachment and proliferation. We have expressed vitronectin in tobacco using both conventional constructs and INPACT constructs. Expression using INPACT constructs has resulted in at least a five fold increase in expression over the best conventional transgenic lines. Further, we have purified vitronection and demonstrated that its characteristics are essentially identical to blood purified vitronectin.

Bioplastic Production Based on Enzyme Engineering: from Microbes to Plants

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Polyhydroxyalkanoates (PHAs) are bacterial polyesters that are produced as an intracellular carbon and energy storage material. PHAs have attracted research interest because PHAs can be processed to useful biodegradable plastic products, such as films and fibres. Our usual life and industry would be indebted to fermentative production of PHAs for environmental merit, realising the ecosystem in which renewable biomass such as sugars and fatty acids are available for making environmentally friendly materials, instead of diminishing fossil fuel. This ecosystem can achieve the microbial conversion from agricultural feedstock biodegradable PHAs derived from CO_2 and water, and finally the product would be broken down again to CO_2 and water.

Rich information obtained from genetically-engineered PHA producing bacteria can be applied to the development of PHA production in transgenic higher plants. The "molecular farming" system based on plants may offer promise of potential success for the cost-effective production of PHAs from carbon dioxide under solar energy. The PHA-producing plants have a potential to reduce the cost of PHA production, because of no cost of feedstock and fermentor settings. Recently, our group developed an *in vitro* evolutionary method that generates highly active mutants of PHA synthases; key enzymes for PHA biosynthesis. Evolved PHA enzymes created through the bacterial screening system, would be effective for efficient PHA production in plants, if such evolvants could be in harmony with metabolic backgrounds of the plant of interest. In fact, increased production of PHAs has been observed in *Arabidopsis thaliana* with evolved mutants.

The presenter will talk about the enzyme engineering for effective bacterial PHA production and its initial application to the photosynthetic PHA production using transgenic plants.

Opportunities and Challenges in Bioprocessing of Transgenic Plant Systems

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The emergence of transgenic crops as alternative protein production systems brings specific opportunities and challenges to upstream and downstream processing. Each plant host contains a unique and variable set of matrix components that must be removed during purification of the target protein. The presence and quantity of these impurities in the extract vary significantly between types of host systems (seed, tubers, and leaves) and extraction conditions. In some cases, extraction conditions such as solid-to-liquid ratio, pH, ionic strength, and homogenization/disintegration method could be used to reduce the amount of host-derived impurities that may interfere with subsequent purification steps. In other situations, specific impurities (e.g. protease, phenolics) may dictate processes conditions and the selection of unit operations. The recent paradigm shift toward the use of contained systems such as aquatic plants and plant cell culture creates additional opportunities but may effect production and downstream cost structure.
Key tasks in bioprocess development are to identify critical process parameters, evaluate their impact on selected recovery and purification steps and rationally design an integrated process that exploits potential advantages of each host system. This presentation will compare strategies currently employed for the downstream processing of seed and green tissue expressed recombinant proteins. Bottlenecks, process cost and developments in recovery and purification technologies for plant-derived proteins will be addressed.

Towards Molecular Biopharming Using Rubber Trees

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The availability of plant expression systems for the production of pharmaceuticals is an important issue in molecular pharming in plants. Pharmaceutical production has evolved from cell culture to highly efficient transgenic systems, which have gained regulatory acceptance. Although the expression of pharmaceuticals has already been reported in microbial and animal systems, the rubber tree has a distinct advantage over many other plant species, as it produces a large amount of latex. When the rubber tree is tapped, the latex exudes from the latex vessels and, thus, transgenic *Hevea* plants can provide a non-destructive system for harvesting recombinant proteins that are synthesized in the latex. Continual production of the recombinant proteins is possible, as the tree can be tapped every other day for its latex throughout its life span of 25 years. Furthermore, since *Hevea* latex is free of animal and human viruses, it can be an important source for the production of therapeutic or healthcare proteins. In addition, the ease of extraction of target protein and large numbers of high expressing rubber plants that can be easily generated without chimaerism through the routine horticultural practice of bud-grafting, make *Hevea* an attractive system. Hence, the transgenic rubber tree becomes a living factory for the production of biopharmaceuticals. Although the strong cauliflower mosaic virus (CaMV) 35S promoter provides ubiquitous expression of recombinant proteins in *Hevea* nevertheless it also enables transcription specifically in the latex of transgenic *Hevea*. Crucially the recombinant protein is expressed in the aqueous latex serum which can be efficiently recovered by centrifugation. Transgenic *Hevea* thus serves as a mini plant factory or bioreactor for recombinant protein production. At MRB, transgenic *Hevea* plants have been expressing GUS protein, a mouse antibody fragment against the coat protein of an oral dental bacterium, *Streptococcus sanguis* and a human protein (human serum albumin) in their latex. From a commercial view point, achieving a high production of recombinant protein is an important factor for the success of using transgenic plants as bioreactors. This may be accomplished by the use of strong promoters such as the latex-specific promoters for hevein as hevein is the most abundant soluble protein in rubber latex.

The Next Step: Product-specific Technical Readiness of Plant-Factories

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Developed with less than the cost of developing a single antibody (± US$ 600M since the 90's), Plant-Factories emerge to serve the next generation of biopharmaceuticals, as well as bio-ethanol, advanced nutrition and other protein-based products.

40 companies and over a 100 research groups worldwide are quietly fulfilling various promises: capacity for ultra large-scale; speed, stability, flexibility and cost-effectiveness; success with difficult to express proteins; pathogen-free products; innovative formulations, etc. Pundits predict a fast decline to big pharmas and to their high-cost, proprietary products; they see the emergence of personalized medicine, generic biodrugs and new therapeutic/diagnostic applications. The disruptive technological advances of plant-factories will play well into these challenging times.

The presentation will challenge the often-cited constraints (growth in open-field, product approval, regulatory of production, financing, glycosylation), and argue that the driver for success and adoption of plant-factories is the "product-specific technical readiness". In other words, protein-factories designed like haute-couture rather than improbable best-in-the-world, one-size-fits-all platforms.

Commercial Feasibility of Plant-Made Vaccines

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Commercialisation of any pharmaceutical or biotechnology product is a complex process with many technical, economic and industrial aspects that can negatively impact feasibility. Successful manufacturing is not in itself sufficient for successful commercialization – facets of regulatory approval, ethical considerations, public acceptance and competitive market forces may each inhibit the introduction and use of new technologies. Plant-made vaccines (PMVs) have attracted much interest from academic researchers and the general public for more than 15 years. The world's first plant-made vaccine was approved for commercial manufacture and use in poultry in January 2006. The development of this vaccine will be briefly described. No PMV for human use has progressed beyond basic proof-of-concept studies. A situational analysis for further development of this technology for human

applications will be presented. A model for assessing commercial feasibility of PMVs comprises detailed evaluation of the technical, economic and industrial aspects; two examples will be described – an oral Hepatitis B vaccine produced in transgenic corn; an intranasal avian influenza vaccine produced in tobacco cell culture.

Production of Biodegradable Plastics in Transgenic Oil Palm

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Malaysia is the largest producer of palm oil in the world and contributing significantly to the world's palm oil production. Currently, the oil palm industry is facing labour and arable land shortage problems, which have forced the industry to increase the return from the same unit area. Genetic engineering with all the promises and advantages was considered and implemented almost 20 years ago for overcoming the above problems. One of the targets for genetic engineering of oil palm is synthesising polyhydroxybutyrate (PHB), a biodegradable plastic. PHB is the most common polyhydroxyalkanoate (PHA) produced as a storage material by bacteria under restricted growth conditions. In bacteria, PHB production is derived from substrate acetyl-coenzyme A by a sequence of three enzymatic reactions: *3-ketothiolase* catalyses the reversible condensation of two acetyl-CoA moieties to form acetoacetyl-CoA. *Acetoacetyl-CoA reductase* subsequently reduces acetoacetyl-CoA to D-(-)-3-hydroxybutyryl-CoA, which is then polymerized by the action of *PHB synthase* to form PHB. Construction of transformation vectors using all the PHB and PHBV genes from bacteria driven by combination of three constitutive promoters, mesocarp-specific promoter or leaf-specific promoter has been carried out. Transformation of oil palm tissues with the various constructs has produced many transformed embryogenic lines where some of them have been successfully regenerated into whole plants and planted in a contained biosafety greenhouse. Molecular analysis, via PCR, demonstrated stable integration of the transgenes into the oil palm genome. HPLC analyses to determine the possible synthesis of PHB or PHBV in oil palm cells is on going. Progress made and problem faced during the study will be elaborated.

Stable Expression of Fibroblast Growth Factor in Plants

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Human Fibroblast growth factors (FGFs) constitute a large family of at least 18 structurally related polypeptides of which FGF-1 and FGF-2 (acidic and basic) are prototype members. FGFs play an important role in cell growth and differentiation, neurite outgrowth, embryogenesis, angiogenesis, and wound healing. More recently, for its implication as the major mitogen in the prostrate cancer, availability of human FGF8b in recombinant form is required for accurate diagnostic tests. To date, plant systems are proposed as heterologous hosts to produce recombinant proteins for use in disease diagnosis and therapy. Here, we describe the stable expression of human FGF8 into *Nicotiana tabacum* cultivar *petit havanna*. The aim of this study was to find an alternative and inexpensive source to produce human FGF8. The HFGF8 cDNA was carried out by a binary vector under the control of the CaMV 35SS promoter and terminator. In addition, an endoplasmic retention signal region KDEL was fused to the C-terminal end of the HFGF8 transgene. For ease of purification, a 6-His tag was added to the 3' end of the HFGF8 cDNA**.** The presence of the transgene in transgenic plants was confirmed by PCR and Southern blots, and the specific transcription of the marker gene in the plants was demonstrated by reverse transcription polymerase chain reaction. Integration of the FGF8b gene into the tobacco genome was confirmed by Southern hybridization. The expression of the FGF8 protein in the transgenic lines was confirmed by Western blot analysis using a polyclonal monospecific antibody to the FGF8 protein. The highest level of expression of the human FGF8 protein in leaves of tobacco was 0.35% of the total soluble protein. The results establish that plants can provide a safe and effective production system for the expression of human fibroblast growth factor.

Molecular Farming of Antimicrobial Peptides and Bacterial Enzymes in Plants

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The global market of enzymes was approximately US$ 1.7 billion (2000) and it has risen to US$ 2.0 billion by 2005. The total market of feed enzymes alone is US$ 150 million. One of the attractive sources of commercially important enzymes is bacteria. The use of thermophilic bacterial enzymes will promote high potential commercialization into two industrial applications, namely biofertilisers and animal feed. Three groups of industrially important enzymes have been cloned and expressed in suitable plant hosts. These enzymes are promising biocatalysts for the large-scale conversion of different types of plant wastes into biofertilisers and value-added animal feed.

The *E. coli* bacterial phytases have been used for the transformation of sugar cane. The recombinant bacterial amylases and cellulases were produced from transgenic plants.

The gene amyE(TV1) was isolated from *Thermoactinomyces vulgaris* 94-2A encoding a nonglucogenic alpha-amylase (AmyTV1). The open reading frame of 483 codons, which was expressed in *Escherichia coli* and *Bacillus subtilis*. Alpha-amylase was found to have 83% homology with the 48-kDa alpha-amylase part of the *Bacillus polymyxa* beta-alpha-amylase polyprotein and 50% homology with Taka amylase A of *Aspergillus oryzae* but only 45% homology with another *T. vulgaris* amylase (neopullulanase, TVA II) recently cloned from strain R-47 (Hofemeister *et al.*, 1994). We manage to produce antibody from the purified protein. The coding sequence of amyTV1 gene was modified by PCR and cloned into *Agrobacterium tumefaciens* binary vector. Alpha-amylases from *Thermoactinomyces vulgaris* was found to be stably expressed in tobacco seeds. Amylase and cellulase enzymes from thermophilic and facultative anaerobic bacteria was cloned from their difficultly growing organisms and then transferred to plant seeds under seeds specific promoters. Both seeds specific promoters USP and legumin from *Vicia faba* was successfully used for expression of bacterial enzymes in plant seeds. The bacterial enzymes were detected by different activity assays and immunodetection assay as well as HPLC analyses of the products of enzyme action on their substrates. Of particular note is the increased thermostability in the bacterial amylase synthesised in plant seeds and the stable activity after storage of dry seeds for one year as well as the stability of the transformation by analyzing the F1 transgenic tobacco seeds. CelZ from the cellulolytic thermophile *Clostridium stercorarium* has been described as a 'monomeric' cellulase able to affect both the endoglucanolytic hydrolysis of internal glycosidic linkages and the exoglucanolytic degradation from the chain ends in a processive mode of action. CelZ protein has a molecular weight of 109 kDa. The production of CelZ directly from anaerobic *Clostridium stercorarium* was difficult. The expression of CelZ in *E. coli* and *B. subtilis* was not sufficient for industrial production. The enzyme CelZ coding region of 3015 bps was modified by PCR and cloned into *E. coli* and *Agrobacterium tumefaciens* binary vectors under the regulation of seed specific promoters and constitutive promoters. Prior to plant transformation, CelZ has been tested to be functional in bacteria. The plant expression cassettes were used for tobacco transformations. The transformed plants were analyzed for CelZ activity. The Molecular mass of CelZ was determined by Western blot analysis. The glycosylation of CelZ in transgenic plants will be shown. The CelZ localized in different plant tissues using different plant promoters and targeting sequences.

Antibiotics are the third largest selling class of drug, with an annual market of between $ 7 billion and $ 22 billion. Current estimates suggest that of this expenditure $ 4 billion to $ 5 billion results from antibiotic resistant bacteria.
Molecular Biofarming of bacterial antibiotics with antiviral, antibacterial and antifungal properties were tried with few successes in transgenic tobacco.

A comparative study of the inhibitory activity of nisin, the well-known lantibiotic produced by certain strains of Lactococcus lactis subsp. lactis, and of the bacteriocin produced by *L. lactis* subsp. cremoris J46, a strain previously isolated from fermented milk, was conducted. For both bacteriocins, the activity against L. lactis subsp. cremoris decreased with increasing pH. In addition, the bacteriocin preparations were more stable at 4 degrees than at 20 degrees C. The influence of the storage temperature was more crucial for nisin. Essentially the same activity was observed for bacteriocin J46 stored for 3 h at 4 degrees or 20 degrees C. More interesting was the observed stability of bacteriocin J46 at pH values between 5.8 and 6.8. For example, about 23% of nisin activity was lost at pH 6.4 whereas no loss of bacteriocin J46 activity was observed (Huot *et al.*, 1996). We found during our project with Bayer AG in Germany. Bacteriocin J46 is simple structure antibiotic compare to Nisin and Subtilin. Bacteriocin J46 coding sequence was synthesized in tube and cloned to *E. coli*. Using Arabinose inducible system Bacteriocin J46 was expressed and expression was detected using antibody epitopes by Western blot analysis. The sequenced approved gene was cloned to *Agrobacterium tumefaciens* binary vectors. Different constructs have been engineered to modify the bacteriocin J46 expression in transgenic plant leaves. We got a lot of transgenic plant carry the mRNA of bacteriocin J46. The mRNA was detected by Northern blot analysis.

Nisin produced by Streptococcus lactis is used as a food preservative and is the most important member of a group of antibiotics containing lanthionine bridges. To understand the genetic basis of these so-called lantibiotics. The nisin structural gene, nisA by (Kaletta C and Entian KD), which is located on a plasmid and codes for a 57-amino-acid prepeptide. The prepeptide is processed post-translationally to the pentacyclic antibiotic. A large number of genes were involved in the regulation, modification, transport and immunity. Till now there is no report about expression in eukaryotes. For eukaryotes nisin expression we assume the *nisA*, *nisB* and *nisC* genes are essential but regulation genes and immunity genes are essential for nisin expression in bacteria. The nisA, nisB and nisC genes were modified by PCR for targeting in ER using plant signal sequence at N-terminus and KDEL at C-terminus. The cloned PCR products was sequenced and cloned in *Agrobacterium tumefaciens* binary vector under the control of 35 CaMW promoter. Tobacco and rape seeds were transformed with Nisin constructs in *Agrobacterium tumefaciens*. The transgenic plants were analyzed at mRNA level using Northern blot and at protein level by Western blot using antisera against NisA. The transcript of *nisA* was detected; the transcript fused to plant signal peptide was bigger than the transcript without plant signal peptide. Although the transcript of *nisA* was small, we have developed the Northern blot technique to detect small transcript. The method for RNA isolation from tobacco was developed for isolation of RNA from rape seeds. NisB and nisC transcripts were detected in tobacco. Other peptides with antiviral and antifungal properties ere cloned and their expression was low but transgenic tobacco showed antiviral and antifungal resistant. The high accumulated mRNA from different insectal and bacterial peptides can explain the reason for antiviral and antifungal resistant.

To utilise the use of tobacco plantation in tropical countries, molecular farming of antimicrobial and bacterial enzymes will bring future possibilities. The conclusion from our experience in previous work the molecular farming of enzymes is much easier compared to antimicrobial peptides.

Agrobacterium-Mediated Genetic Transformation of *Psorales corylifolia* by ADC Gene and Quantification of Psorelen and Diadzein through HPLC

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The establishment of plant genetic engineering techniques coupled with the emerging details of various metabolic pathways have made it possible to target and engineer secondary metabolite biosynthesis. These genetically engineered cells or tissues can then be made to regenerate into whole plants or be stably maintained in bioreactors. for increasing product levels, targeting the branching point between primary and secondary metabolism seems to be a better candidate for initiating metabolic engineering.

In the present investigation we have tried to develop a system where the genetic modification will lead to increased production of a target secondary metabolite. Transgenics of *Psorales corylifolia*, an endangered medicinal plant which is the commercial source of psoralen and diadzein, have been developed by us. Arginine decarboxylase (ADC) which is involved in the biosynthesis of polyamines, putrescine, spermine, spermidine, and also in the biosynthesis of secondary metabolites has been over expressed in *Psoralea.*

Nodal segments obtained from field grown plants and callus pieces obtained from cotyledonary leaves, hypocotyls and radical segments were transfected by *Agrobacterium tumefaciens* LBA 4404 carrying *adc* gene under the control of CaMV 35 S promoter .Various parameters such s type of explants, duration of co cultivation, bacterial cell concentration as well as co cultivation medium were tried to ascertain the best response. Best response was obtained with callus derived from juvenile cotyledonary leaves on MS + NAA + Kn + 2,4 D after 15 minutes pre-incubation and 36 hr co cultivation The co cultivated callus was grown on selection medium containing Kanamycin. After 3 weeks of selection, Kanamycin resistant shoots were developed. The transgenic lines (callus lines) exhibited better growth performance in terms of early and maximum (100%) regeneration with good quality shoots than the non-transformed lines. Different levels of psoralen and diadzein were recorded through HPLC in non-transformed and transformed lines.

A Plant Virus Based Expression System for Hepatitis B Surface Antigen

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The *Cucumber green mottle mosaic virus* strain SH (CGMMV-SH) is very well characterised, therefore making it an attractive candidate for the development of a new chimeric virus based expression system. In this study, a novel CGMMV full-length clone was constructed and infectious transcripts derived from it were found to have the same degree of infectivity as purified naked viral RNA. This high efficiency clone was then used to construct a chimeric vector where the coding DNA sequence

of the "a" determinant of Hepatitis B surface antigen was coupled to the end of the CGMMV coat protein gene via a leaky UAG signature sequence. The coat protein composition of the chimeric virus derived from the vector consisted of recombinant fusion coat proteins which have the "a" determinant of the HBsAg fused to the coat protein, and recombinant CGMMV coat protein at a 1:1 ratio.

The expressed "a" determinant of the HBsAg could be detected using commercial HBs ELISA based diagnostic kits indicating that the expressed HBsAg retained its immunological properties. Introduction of the chimeric virus into the Peripheral Blood Mononuclear Cell (PBMC) culture showed 3 fold increase in *in vitro* HBs specific antibody production by the PBMC. This also suggests that the expressed "a" determinant of the HBsAg could potentially be a good vaccine candidate.

The leaky UAG signature sequence used is this study produced approximately 50% read through efficiency which is higher than the previously reported 10% read through efficiency. Later MALDI-ToF analysis revealed that the read through was caused by $tRNA^{leu}$.

Prospects and the possible applications of the vector system developed in this study are also outlined.

Potential of Algae as a Biopharming System

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Algae are commonly used as human food, feed, fertilizers and as sources for industrial gums and chemicals, mainly, the phycocolloids. Algae could also be a promising source for novel bioactive compounds as it was reported that algae used targeted antimicrobial chemical defence strategies. In addition to natural resources, recombinant technology offers an alternative to optimize the exploitation of algae by creating or introducing new uses to the algal industries, especially in the production of value-added algae. Algae can be genetically engineered to contain a gene sequence that produces the desired protein such as health-promoting secondary compounds, nutraceutical and pharmaceutical products including "edible vaccines". Cell suspension of these algae can then be cultured to biosynthesis novel biomedical compounds under a controlled environment. Thus, the development of algal gene manipulation system; together with the development of algal cellular technology will definitely enhance the potential use of algae as a biopharming system.

Comparative Studies of Sulphur Utilisation Efficiency of Indian Mustard Genotypes

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Fourteen genotypes of mustard [*Brassica juncea* (L.) Czern. & Coss.] were grown for 30 days in complete nutrient solution with 50 mM (S-insufficient) and 1 mM (S-sufficient) sulphur levels, and sulphur efficiency (SE) was analysed. SE, the ability of a plant to maintain good growth on S-limited condition, was calculated as the ratio of dry matter production at low and high S supply. There was 2-fold to 10-fold differences in SE of the genotypes. Of the 14 genotypes, Pusa Bold was identified as the most S-efficient, while Pusa Jai Kisan the most S-inefficient. SE of Pusa Bold was about 10-fold higher than Pusa Jai Kisan. To find out the physiological basis of this difference, we investigated the possible role of SO_4^{2+} influx across the root cell plasma membrane in conferring SE by measuring the short-term $^{35}SO_4^{2-}$ uptake in two contrasting genotypes, S-efficient Pusa Bold and S-inefficient Pusa Jai Kisan. This was done by quantifying sulphate uptake over two different concentration ranges: a high-concentration range (100-500mM) and a low-concentration range (5-50mM). Uptake experiments revealed the presence of two separate sulphate transport systems mediating high- and low-affinity sulphate uptake. Interestingly, the sulphate uptake by the roots of Pusa Bold is mediated by both high- and low-affinity transport systems, while that of Pusa Jai Kisan by only low affinity sulphate transporter system. The study suggests that root SO_4^{2-} uptake rate may play an important role in conferring S efficiency of mustard genotypes under S-limiting condition. This information may be useful in developing mustard varieties more efficient in acquiring S from soil solution through genetic manipulation.

Advanced Bioreactor System for the Production of Fragrance Compounds from *Michelia alba*

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Michelia spp. is known to produce high value essential oil for the perfumery industry. The essence of the world's most expensive perfumes, such as JOY and J'adore, is based on the oil of *Michelia* spp. Studies were conducted to develop an efficient cell culture system for the production of callus, suspension cell cultures and somatic embryos from *Michelia alba* and for the production of essential oils using advanced

bioreactor technology. Direct somatic embryogenesis has been successfully obtained from petal explant using MS media containing 2,4-D. On the other hand, MS media containing BA produced both callus and globular structures of somatic embryos within 10-15 days of incubation. Seven developmental stages of *Michelia alba* ('Cempaka putih') flowers namely Stage 5 (S5) to 11 (S11) were investigated for their volatile constituents. The essential oil was isolated by Simultaneous Distillation Extraction (SDE) technique and the oil obtained was subjected to GC-MS analysis. In total, seventy-seven compounds (77) representing 93-98% of the overall *M. alba* volatiles were identified on the basis of mass spectra and retention indices. Thirty-three (33) of these compounds belonged to the isoprenoids group which comprised 30-50% of the total volatile compounds whereas the remaining belonged to fatty acid derivatives, benzenoid, phenylpropanoid and other hydrocarbon compounds. One major problem anticipated in this approach, based on our early experiments, is limited or minimal amount of fragrance produced in the cultures. The appropriate strategy is to superimpose DNA microarray studies on top of the cell culture project. The study, which will also cover the natural flower development phases, will lead to the identification of genes or sets of genes that regulate for the production of the fragrance.

Development of Bioreactor System for Propagation of *Eurycoma longifolia* spp. (Tongkat Ali) Cells

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Eurycoma longifolia spp. (Tongkat Ali) is widely cultivated in the tropical region especially in Malaysia, Indonesia, Thailand and Vietnam. The root of the plant is well known to exhibit medicinal properties and produces many chemical compounds such quassinoids, canthine and its derivatives like squalane, tirucallane, and triterpenes. The active compounds are reported to have antimalaria properties, cytotoxic, aphrodisiac and anti-ulcer activities. This species are normally propagated by seed and in vitro propagation using explants. Propagation of plant using seeds takes a long time, however propagation of explants using tissue culture such as induction of callus of the plants are becoming popular. Development of bioreactor system for cultivation of the plant tissue cells provide an alternative method for propagation of these cells in a much faster way compared to the traditional method of seedling plants. Important bioreactor parameters for tissue culture propagation have to be optimized for optimum cell growth. Case study using shake flasks and airlift bioreactor system on Tongkat Ali tissue cells will be shown and discussed in our study here.

Plant-based Molecular Farming for Vaccine and Adjuvant Production

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Over centuries, plants have been used for the production of medicinal compounds and one quarter of prescribed drugs are still of plant origin. Advances in genomics and proteomics resulted in identification of exponential number of useful genes. It is unlikely that a single expression system could be utilized to express these genes. Plants have been used as novel expression systems, which enable safer and economical production of pharmaceutically important proteins. There is a growing interest to develop oral vaccines and mucosal adjuvants as it is the most preferred and effective means to attain mucosal immunity against most of the diseases. Vaccines and adjuvant production in edible plant tissues may enable the successful introduction of oral/edible vaccines in the mass immunization programs of the developing world. Hepatitis B continues to be an alarming disease in the developing countries, because of the prohibitive costs of the available vaccine. Oral plant based vaccine with co administration of a mucosal adjuvant may provide an effective and affordable strategy to control the disease spread.

We have successfully expressed hepatitis B surface antigen (HBsAg) in tobacco plant and cell cultures, soybean cell cultures, microtubers and hairy roots of potato and transgenic banana and tomato fruits. The expression of HBsAg was confirmed by molecular analysis and characterized for its biophysical properties in terms of buoyant density. Attempts were also made to enhance the expression levels using different expression cassettes, by wounding and/or treatment with plant growth regulators. The immunogenicity of HBsAg is dependent upon co-administration of adjuvants. A non-toxic mutant of heat labile enterotoxin of enterotoxigenic *E. coli* (LTK63) is an excellent mucosal adjuvant and its adjuvanticity has been demonstrated using a wide range of antigens. A synthetic bicistronic gene encoding LTK 63 with a banana preferred codon usage has been constructed and it was expressed in yeast, tobacco, potato and banana. The expression of LTK 63 was confirmed by molecular analysis. Our results on these aspects will be presented.

Hairy Root Culture as a Source for Pharmaceuticals

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Plants are a major source for natural products such as pharmaceuticals, agrochemicals, flavour and fragrance ingredients, food additives, and pesticides. Several distinct plant-derived chemicals have already been used as important drugs in one or more countries in the world. From these comes the more simple synthetic modifications of the naturally occuring drugs. For the discovery of new products of medicinal value for drug development, traditional agriculture has been aggresively supported by many modern biotechnological tools, such as plant tissue culture techniques and metabolic engineering, especially in the production of bioactive plant metabolites. Different strategies, using an in vitro system, have been extensively studied to improve the production of plant chemicals. For example, cell suspension culture system offers large scale culturing of plant cells (either continuous or in batches) from which secondary metabolites could be extracted. Alternatively, hairy root cultures (either transgenic or otherwise) have revolutionised the role of plant tissue culture in secondary metabolite production. The hairy root cultures are unique in their genetic and biosynthetic stability, exhibit faster growth rate, and can be easily maintained. This leads to the opportunities for high volume production of pharmaceuticals, nutraceuticals, and other beneficial substances. Besides being an attractive alternative approach to traditional methods of plantation for raw materials, it offers a more controlled supply of biochemicals independent of plant availability. This is the case in our study, where we have initiated a hairy root culture system for an important medicinal plant. The hairy root culture system developed has also been shown as an important source for the extraction of several compounds having potentially high pharmaceutical value.

Effective Assessment and Substantiation of the Supply Chain Management System to Ensure GM Food Safety in India

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Ten years since GM crops were first introduced for commercial cultivation, many countries including India, still debate as to the safety issues in cultivating them. India has a positive outlook towards GM crops yet maintains a cautious attitude because of the possible biosafety concerns. Stringent regulatory norms for production, cultivation and distribution of GM food crops have not yet been concretized. The applicable norms (for Bt cotton, which is the only GM crop to be cultivated in India) pertain only to a non-food crop. Agbiotech pertaining to food crops is gaining importance among the general public in India more for its associated controversies than for the perceived benefits accruing to developing countries, in general. The layman's understanding of GM crops/food focuses mainly on ethical issues and food safety aspects. The policy makers focus on socio-economic effects and impact on biodiversity without effective communication about the beneficial aspects.

Global food safety standards are undergoing continuous evaluation and monitoring with the FAO/WHO/CODEX Committee taking the lead in implementing the principles. Any transgenic crop is subjected to risk assessment tests based on certain set measurement standards on a case-by-case basis. The comparative approach based on substantial equivalence is being supplemented with newer targeted and non-targeted approaches.

There is a lack of resolve within the Indian establishment to regulate GM food safety. India is favoured for the high profit margins, poor legal and environmental regulations and powerful lobbies that focus only on the business aspects of GMO. The end result is a tangled informational jumble from which is extricated only the negative prospects of cultivating GM crops.

The regulatory scenario in India involves a multiplicity of agencies to monitor cultivation and release of GM crops. Definite data related to safety studies are not available in the public domain. Our paper focuses attention on the supply chain system from the supplier through the farmers, the cultivation approaches, harvesting process, marketing, post-market surveillance and consumer acceptance of the genetically modified food product or products containing GM ingredients as applicable to India, where there is a lack of systematic co-ordination. Intrinsic (food safety, nutritional attributes, sensory, value and process attributes) and extrinsic (test indicators like applied quality management systems, certification, minimum quality standards set, brand, brand perception, country of origin) parameters are integrated to develop a comprehensive standard modus operandi relevant to any type of GM food crop.

Patent Issues in Molecular Farming

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Kass International Sdn.Bhd.

Intellectual property (IP) is the property resulting from the creative or "inventive" work of an individual or individuals. The protection of IP is described in a large body of law that includes copyrights, trademarks, trade secrets, and patents. Why Intellectual Property is important? Intellectual Property (IP) protection, more specifically the patent system is important to provide incentive to an industry for investing in developing an innovation. Moreover, IP protection is needed in molecular faming due to the time and effort invested in development a new plant pharmaceutical products. However, IP protection as well as good IP management has its important value towards the development of molecular farming products. The strengthening of IP protection for molecular farming should be gained through patent licenses agreements, material transfer agreements and technology transfer agreements.

Pharmaceutical Crops: A Legal Perspective on its Prospects for Commercialisation.

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The commercialisation prospects of plants being used as delivery methods for pharmaceuticals displays great potential, especially with the enormous strides that science has made in the past decade or so. However, when a legal perspective is adopted, the commercialisation prospects of pharmaceutical crops look far less promising.

The regulatory framework relating to pharmaceutical crops in the European Union (EU), and the *Cartagena Protocol on Biosafety to the Convention on Biodiversity* (henceforth the Protocol) will be examined, analysing its workability in regulating pharmaceutical crops so that only those crops which are safe for the environment and human health are commercialised. The discussion focuses on the growing and marketing of pharmaceutical crops, and the issues of intellectual property will not be addressed. The criticisms of both legislative frameworks will also be drawn out. Essentially, the EU framework lacks specificity and the law regulating pharmaceutical crops is governed by two areas: the regulation of genetically modified crops, and the regulation of pharmaceuticals. This has resulted in many gaps in the EU law, whereas the Protocol is lacking in terms of its limited scope and power of enforcement. Thus, it is suggested that neither the EU nor the Protocol legislative frameworks will adequately ensure the safety of a pharmaceutical crop before they are commercialised. The ideal solution would be to develop a detailed framework of efficacious legislation that specifically addresses the exclusive concerns of pharmaceutical crops. Thus, this paper concludes that until and unless such comprehensive regulatory systems are put in place, the bold forays of pharmaceutical crops into the commercial arena may never become a reality.

Biochemical and Cellular Studies of Plant Defence Lignification Response for *Fusarium oxysporum cubense* race 4 (Focr4) Resistance ('Jari Buaya') and Susceptible ('Rastali') Cultivars

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Lignin is a term used to define a complex group of phenolic polymers that strengthens the plant cell wall. In addition to lignification associated with plant biological development processes, the accumulation and deposition of phenolic compounds, lignin also underpins defence responses. It has been proved that this response plays a crucial role in non-host or passive resistance. Once it reaches the desired location, lignin polymer is formed through esterification or etherification. Lignin deposition is always correlated with a surged activity of enzymes in phenylpropanoid pathway during pathogen infection regardless if it is a compatible or incompatible plant-pathogen interaction which causes the hypersensitive response through the formation of necrotic lesions containing highly lignified cells around the pathogens. However, limited information is currently available for the comparison of lignification related properties of susceptible and resistance plants' enzymes in phenylpropanoid metabolism. This study was therefore conducted with banana tissue culture plantlets to ascertain the activity of phenylalanine ammonia lyase (PAL), phenolic and lignin contents in resistance ('Jari Buaya) and susceptible ('Rastali') banana cultivars through biochemical and histological assays. 'Jari Buaya' possessed higher amount of phenolic compounds for both the intracellular and wall bound types. However, susceptible cultivar, "Rastali" secreted extra phenolic compounds into cultured media more than the resistance cultivar. The metabolic studies of phenolic compounds were in tandem with the PAL enzyme activity and specific histological study for lignin detection which unveiled that

resistance cultivar possessed higher PAL activity and more lignin deposition in the roots. This is a detailed report of the globally renowned Focr4 resistance and susceptible local crops, 'Jari Buaya' and 'Rastali' regarding the biochemical and cellular properties, if there is any possibility of synchronising effects for both active and passive defences during pathogen attack.

Establishment of Cell Suspension Cultures of *Aquilaria malaccensis*

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Aquilaria malaccensis is a tropical forest tree of the family *Thymelaeaceae*. This tree produces agarwood or gaharu, a highly valuable, resinous and fragrant forest product. Agar wood has been highly recognised for it's vast medicinal values and widely use for perfumery, incense and religious purposes. Currently, the high value of agarwood stimulates illegal harvesting and as a result, *Aquilaria* trees are often cut down indiscriminately. Populations of this tree have declined and may lead to possible extinction in the near future. In addition to conserving the trees and ensuring sustainable supply of the agarwood, methods for propagation need to be developed. This study aims to look into the micropropagation system of *A. malaccensis* via cell suspension cultures. A reproducible system for callus initiation and establishment of cell suspension cultures is being studied and optimised. In this study, young leaves of *A. malaccensis* were cultured on Murashige and Skoog (MS) medium with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP). Within 3 weeks, friable embryogenic callus was developed in MS medium supplemented with 2 mg/l 2,4-D + 0.5 mg/l BAP. Friable callus produced was transferred into liquid MS medium supplemented with the same combination and maintained in the dark. It was found that after one month, suspension cultures were successfully established and showed good proliferation and formation of new cell aggregates.

Shoot Regeneration of *Aquilaria Malaccensis*

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The agarwood tree *Aquilaria malaccensis* from the family *Thymelaeaceae* is well known for its oleoresin, a secondary metabolite product produced in the terpenoid biosynthesis pathway. Micropropagation via tissue culture technique was carried out to develop shoot regeneration in *A. malaccensis*. Medium ½ MS was used to germinate the seeds. Shoot regeneration and elongation were carried out in medium MS supplemented with 0.3 mg/l Benzylaminopurine. As a result, multiple shoot induction was obtained in medium MS supplemented with 0.3 mg/l Thidiazuron. Due to the high demand of *A. malaccensis* seedlings in local market, the *in vitro* micropropagation technology is established to supply the seedlings for market need.

Towards Production of High Palmitoleate Transgenic Oil Palm

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One of the targets for oil palm genetic engineering is increasing palmitoleic acid. Palmitoleic acid is produced by desaturation of palmitic acid by a specific 9-ACP desaturase, which acts selectively on palmitic acid as substrate to produce palmitoleic acid. Vectors carrying two types of mutant desaturase genes driven by constitutive promoters and oil palm mesocarp-specific promoter have been constructed. Plastid targeting sequence (transit peptide; TP) has been fused to 5'end of the open reading frame of these genes to ensure the expression is targeted in the plastid. Matrix attachment region of tobacco (RB7-MAR) was also included, with the hope of stabilising transgene expression and minimising silencing due to position effect. Transformation of these vectors into oil palm embryogenic calli has just initiated. Progress made to date will be elaborated.

The Complete Nucleotide Sequence and Genomic Organisation of *Cucumber mosaic virus*

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Plant viruses are unable to replicate in human beings; making them an attractive system for the expression of foreign proteins in plants. Thus, virus vectors can be used as carriers for the development of innovative vaccination strategies. One candidate being developed in our laboratory is the *Cucumber mosaic virus*. *Cucumber mosaic virus* (CMV) is one of the most widespread plant viruses. Up to now, more than 60 strains have been reported. We are interested in characterising the sequence of the Malaysia strain (C-strain), and its evolutionary relationship with other CMV viruses. Full-length cDNA of CMV RNAs 1, 2 and 3 were constructed from total viral RNA through RT-PCR and cloned downstream of a bacteriophage T7 promoter in plant. The three genomic RNA nucleotide sequences were designated RNA 1 (3.3kb in length), RNA 2 (3.0kb) and RNA 3 (2.2kb). The genomic organisation was found to be similar to other cucumoviruses. The 5' non-coding region, open reading frames and 3' non-coding region of RNA1, 2 and 3 were compared with published CMV sequences.

Biological Analysis of Chimeric CGMMV Expressing HBsAg 'a' Determinant Peptides

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Hepatitis B virus (HBV) infection is a major health problem in many developing countries. With over 350 million people worldwide infected with HBV, there is a great need for an inexpensive vaccine that would allow vaccination of large segments of the population. Today, there is increasing interest in the immune response induced by chimeric plant viruses that possess recombinant coat proteins carrying antigenic oligopeptides. Plant viruses are non-pathogenic to humans, free from mammalian pathogens, are simple systems for expression and purification, cost-effective and amenable to large-scale production. In addition, the employment of plant viruses for the production of therapeutic proteins and vaccine delivery system offers several advantages over conventional bacterial or animal virus systems. Recently in our laboratory, a chimeric *Cucumber green mottle mosaic virus* (CGMMV) expressing Hepatitis B surface antigen (HBsAg) was developed using molecular techniques. The gene encoding HBsAg '***a***' determinant was joined to the 3'-terminal of the viral coat protein gene via a sticky amber stop codon. The rod-shaped CGMMV particles are built up of many copies of identical coat protein subunits, thus the HBsAg '***a***' determinant (34 amino-acid peptide) that is engineered to fuse to the C-terminal of the coat protein will be presented in multiple copies. In this study, the infectious recombinant RNA genome transcript was inoculated onto muskmelon seedlings and the chimeric virus particles was successfully purified from leaf tissues of the plants after three weeks. The chimeric virus was confirmed using RT-PCR and enzyme-linked immunosorbent assay (ELISA), and the antigenicity of the expressed HBsAg peptides was tested using two commercially available HBsAg clinical diagnostic kits. Positive results were observed where the HBsAg peptides on the CGMMV coat proteins have been clearly detected in both of the *in vitro* immunoreactive assay kits. These results demonstrated that the chimeric CGMMV was misidentified as 'HBV' or 'HBsAg'. Thus, we concluded the HBsAg '***a***' determinant peptides have been correctly expressed and displayed on the surface of the CGMMV, and have also retained its immunological properties. We have also carried out a preliminary experiment to see whether or not the chimeric CGMMV could elicit specific antibody production in mice. Results have shown that the chimeric CGMMV elicited specific serum antibody production in mice as measured by ELISA. In addition, the purified chimeric virus has shown a systemic immune response in BALB/c mice with the absence of adjuvant. However, further work must be carried out in order to study in depth the humoral and cellular immune response in mice following immunisation with the chimeric CGMMV. We are hoping that this system would be widely implemented in human vaccination programs against HBV or other pathogenic microbes in the near future.

Enhancement of Primary and Secondary Metabolite Contents in the Callus Culture of *Hyoscyamus niger* L. by NaCl Stress

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India is a large repository of genetic resources. It is rich in biodiversity. It occupies only 2.4% of the land area of the world but its contribution to global biodiversity is 8%. Medicinal plants which constitute a segment of flora provide raw material for use in all the indigenous medicinal systems. The increasing world demand and renewed global interest in traditional ethno pharmacy coupled with increasing preference

for natural substance in the healthcare system, the natural stock of medicinal plant is under tremendous pressure. Plant tissue culture will help in generating large number of plantlets throughout the year. In addition, through *in vitro* culture technique effect of growth regulators and biotic and abiotic yield can be enhanced.

In this present investigation we have tried to raise callus from the explants of *Hyoscyamus* on MS+ NAA+ BAP+ CH. The compact and green callus was transferred to an MS medium supplemented with varied concentration of NaCl (0-200 mM). *Hyoscyamine* quantified through HPLC in callus on salt fortified medium exhibited variation. Maximum hyoscyamine was recorded at 75mM NaCl. Protein and proline accumulated as a consequence of salt stress.

Evaluation of a *Cucumber green mottle mosaic virus* Fusion and Read-through Expression Vector

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Plant virus vectors have been shown to provide useful transient expression systems for the production of peptides or antigenic epitopes in plants. *Cucumber green mottle mosaic virus* (CGMMV) is another potential candidate for such use. Theoretically, this plant virus system can be used to express any small peptide on its surface provided the immunogenic epitope gene sequence is known. In this study, fusion and read-through expression constructs were formed using the CGMMV coat protein (CP) in a previously constructed CGMMV transcription vector. It was shown that the fusion expression system was not suitable for direct fusion of the foreign peptide downstream of the CGMMV CP. The read through expression strategy was found to be the solution for this limitation. Apart from the limitation on size of inserted peptides and usage of leaky UAG amber stop codon, the pI:charge can affect the chances of obtaining viable recombinant virus. The ratio of modified to unmodified CP was found to be approximately 1:1 and confirms a similar ratio obtained in a previous study. Sequence analyses also revealed that foreign peptide expressed via the leaky CGMMV specific UAG amber stop codon on CGMMV CP enabled the virus to move systemically within the host plant. Removal or deletion of the inserted peptide was shown to occur in the 5' to 3' direction. This led to the partial expression of inserted peptide preventing the detection of the expressed peptide through immunodetection methods.

In vitro Transcription and Expression of Dengue 2 Virus Antigen on *Cucumber green mottle mosaic virus* Coat Protein Surface

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Plant virus vectors have been developed and tested for their use as antigen presentation systems and for the production of foreign peptides with biopharmaceutical properties. In this study, Cucumber green mottle mosaic virus (CGMMV) was used to present a truncated dengue 2 virus envelope (E) protein binding region number four, EB4. The EB4 was inserted downstream of the coat protein (CP) open reading frame (ORF) of a CGMMV transcription vector. Using the same approach, fusion recombinant and read through recombinant clones were constructed. All the recombinant transcription clones were transcribed into mRNA to obtain capped full length recombinant CGMMV transcripts. The transcripts were then used to inoculate plants susceptible to CGMMV. The inoculated plants were then monitored for growth and appearance of symptoms. Viruses were isolated from infected plants and SDS-PAGE analysis showed the presence of coat protein band. Downstream characterisation and immunological studies of the purified virus are in progress.

Exploring *Hibiscus chlorotic ringspot virus* as a Plant Viral Vector

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A recombinant *Hibiscus chlorotic ringspot virus* (HCRSV) as an expression viral vector was investigated for the expression of a broad spectrum anti-microbial peptide, Caerin 1.1. The recombinant HCRSV vector, pHCRSVsg2, contains a duplicated, homologous, subgenomic promoter 2 inserted into the coat protein (CP) coding region. This disruption prevents expression of the HCRSV coat protein and thus inhibits systemic infection of kenaf (*Hibiscus cannabinus* L.) plants. The kenaf protoplast system was thus chosen to investigate the ability of pHCRSVsg2 to replicate and express

the Caerin 1.1 protein. Successful construction of an infectious pHCRSVsg2CaeF clone was constructed, as affirmed by northern blot analysis. Using Coomassie blue staining and western blot analyses, detection of Caerin production in kenaf protoplasts could not be achieved, possibly due to the absence of or the inadequate expression of the proteins of interest by the kenaf protoplast system.

Synthesis of a Full-length cDNA Clone of *Zucchini yellow mosaic virus* – Singapore Isolate

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The complete genome of the Singapore isolate of *Zucchini yellow mosaic virus* (ZYMV-S) consists of 9603 nucleotides and it encodes a polyprotein of 3802 amino acids with a calculated molecular weight of 350 kDa. This polyprotein precursor undergoes further auto-proteolytic processes to yield mature proteins. Through the synthesis of a full-length cDNA clone of the ZYMV-S, viral-host interactions and specific functions of these proteins can then be examined and possibly for further development into an expression vector.

Attempts were made to construct full-length cDNA clones of ZYMV-S in this study, through the use of eight PCR primers designed based on specific virus sequence. The sizes of the PCR fragments were 1.8 kb, 3.2 kb, 1.7 kb and 3.5 kb. All fragments were successfully synthesised and subsequently cloned into pGEM-T Easy vector to allow for further manipulations. One full-length cDNA clone was obtained from later work.

Investigations on the full-length cDNA clones, based on restriction enzyme screening and DNA sequencing, confirmed that the cDNA fragments were ligated in the correct orientation. However, understanding that random mutations may have been introduced into the cDNA fragments during the PCR process which may result in point mutation, further investigation is required to ascertain the sequence integrity and biological activity of the full-length cDNA clone obtained.

Development of *Cymbidium mosaic virus* as a Viral Vector for Biomedical Applications.

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Plants are commonly used in foreign protein expression. This can be achieved via genetically transformed plants and transient expression through viral vectors. The latter requires plant viruses as delivery systems to express the gene of interest in the host plant. High and rapid yield are obtained from the transient expression strategy, making it a suitable candidate for possible large scale vaccine production. Currently, a biologically active cDNA clone of *Cymbidium mosaic virus* is available for development into such a protein expression vector. Previous work has shown that gene insertion might have disrupted the subgenomic promoter sequence of the viral CP gene, thus affecting virus infectivity. Mutagenesis studies were performed on sequences upstream of this gene to locate the vicinity in which the promoter lies. Attempts were also made to construct a duplicated subgenomic promoter for possible vaccine production.

Production of Recombinant Protein of Newcastle Disease Virus (NDV) in *Centella asiatica*

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Plant biotechnology and genetic engineering have led to the production of various pharmaceutical proteins from plant sources. Plants are potential biofarming features because they provide an inexpensive and convenient system for the large-scale production of valuable recombinant proteins. Over the past decades, Newcastle disease virus (NDV), a member of the *Paramyxoviridae* remains to be one of the most threatening diseases of poultry worldwide. Haemagglutinin-neuraminidase (HN) protein, one of the NDV envelope proteins, is responsible to induce immune response of the host prior to NDV infection. This potential recombinant protein can serve as an alternative antigen in triggering the host immune responses against NDV and contribute to the control of the disease. Thus a study was carried out to clone the HN gene of NDV strain AF2240 into plant expression

vector (pCambia 1302) carrying the GFP and driven by the CaMV 35S promoter. The construct was bombarded to the plant cell system through a biolistic gene gun. Then, expression of the HN protein was evaluated by monitoring the GFP expression and further confirmed by molecular approach. This study will help the production of low cost NDV vaccine in plant cell culture systems and control the NDV disease in poultry, thus preventing heavy economic losses worldwide.

openSputnik – a Plant Genomics EST Analysis Database and Processing Pipeline

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Many plant genomes are significantly larger than even the human genome. In order to understand more about the gene toolbox of plant species, various large-scale genome-sequencing projects have been successfully launched e.g. *Arabidopsis thaliana*, *Oryza sativa* and *Populus trichocarpa*. The preparation of cDNA and large-scale expressed sequence tag (EST) sequencing remains a dominant genome sampling methodology in many laboratories. ESTs provide a cost-effective way to obtain sequence information from the genes transcribed in the individual species. To date, the number of plant ESTs deposited in the public databases exceeds 9.3 million sequences from over 320 species, and additional data continues to be deposited for an increasingly broad coverage of the plant kingdom. Major plant ESTs sequence collections are concentrated primarily to the higher plants (Angiosperms) with little attention paid to lower plants. We are working on several species of lower plants (Bracken fern, Merlin's grass and Hornwort) and the EST sequencing data collected will be analysed with an EST analysis pipeline called openSputnik. The openSputnik has been designed as a platform for the comprehensive annotation and analysis of EST sequences in a comparative and taxonomic context. The EST sequences are followed by an exhaustive annotation pipeline. This results in a clustered and assembled 'unigene'-set and a collection of probable peptide sequences and their functional and structural attributes. The resulting peptide sequences are placed in context with the currently available complete plant genomes and compared to other clustered EST collections. The openSputnik database, as a result, creates a platform upon which the patterns of general housekeeping genes and lineage-specific gene families may be teased apart.

Preliminary Study on Stability of Anti-Cucumber Mosaic Virus (CMV) Single-Chain Antibody (scFv) Expression Level in T_1 Transgenic Plants by Real Time-Polymerase Chain Reaction

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Cucumber Mosaic Virus (CMV) is a significant plant pathogen affecting various crops and plants in Malaysia. It is a type of RNA virus and exists as a number of allied strains. As the virus infection is widely spread in many economically important crops or plants, the production, cloning, expression and analysis of these antibodies in local agronomically important plants will not only produce potentially resistant varieties but also enhance development of antibody and transgenic plant technology and give insights into mechanisms of viral pathogenecity and plant resistance among the plant species. A single chain variable fragment anti Cucumber Mosaic Virus antibody was synthesised via a scFv library constructed with mRNA from the spleen cells of a CMV coat protein-immunised mouse and transformed via *Agrobacterium tumefaciens* into tobacco plants, *Nicotiana tabaccum* L. cv. White burley. In this study, T_1 transgenic plants were analysed for expression stability by real-time polymerase chain reaction using actin as the endogenous control house-keeping gene.

Expression of Dengue Viral Protein in Plant Systems

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The project is divided into 2 sections; DNA cloning and plant transformation. The prerequisite step in DNA cloning was to clone a dengue 3 (DEN3) DNA fragment (1.8kb) into pEAN2301 plasmid (modified plasmid from pCambia2301 and pBI121), a plant transformation vector at the *Bam*HI site to give pDEN2301 plasmid. The pEAN2301 plasmid contains a construct of 35S' promoter gene and selectable marker gene (NPT11) (neomycinphosphotransferase) in the T- DNA region. In order to determine the presence of the DEN3 DNA, this plasmid was verified by digestion with *Bam*HI, polymerase chain reaction (PCR) using DEN3 sense and antisense primer, and DNA sequencing was carried out. By using electroporation, this new plasmid was then transformed into *Agrobacterium tumefaciens* (strain LBA4404) before transforming into plant system.

For plant transformation, tobacco plant (*Nicotiana tabacum* L. var Samsun) used as the plant system. Transformation of tobacco plants was carried out using the co-cultivation method. The putative transgenic plants were selected on MS media containing 100mg/l kanamycin. The biochemical analysis of the transgenic plants was done using polymerase chain reaction and Southern blot. Interestingly, positive results were observed in both experiments, which strongly indicated that the dengue virus gene was successfully integrated into the tobacco plant genome. The expression of dengue viral protein in tobacco was determined by ELISA analysis and a putative positive result was obtained.

The production of dengue viral protein in plant system opens an avenue for an alternative source of dengue virus protein instead of using animals. This protein is used in dengue test kits for dengue infected patients.

RNAi : Good Tool For Metabolic Engineering in Plants?

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RNA interference (RNAi) has been used widely in a variety of organisms, including plants and animals. RNAi is a post-transcriptional gene silencing process in plants and involves mRNA degradation. It is a powerful tool for creating gene-specific phenotypes of loss-of-functions mutations which also prove to be a good tool for metabolic engineering. In this study, RNAi has been applied for 2 genes, *CHS* which involves in the anthocyanin accumulation and *SFR3* gene which involves in producing metabolite for cold tolerance in plants. For *CHS* gene all 48 transformants have failed to accumulate anthocyanin in respond to high light treatments and for the *SFR3* gene, 7 out of 48 transformants have failed to show the cold tolerance mechanisms with a different degree in silencing. In conclusion, the RNAi technique is suitable as a tool to manipulate metabolite in any biological pathway in plants.