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Poster Abstract – F.01

THE SEEDS OF HOPE

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autoimmunity, diabetes mellitus, GAD65, oral tolerance, transgenic plants

Type 1 insulin-dependent diabetes mellitus (T1DM) is caused by autoimmune destruction of insulin-secreting β cells and afflicts 0.2-0.3% of the population. The young age of affected patients, the need for life-long insulin therapy and the high prevalence of late-onset complications make this disease an enormous health problem. The smaller isoform of glutamic acid decarboxylase of 65 kDa (GAD65) is a major autoantigen in human T1DM. Induction of oral tolerance has been reported to modify the natural history of several autoimmune diseases both in experimental models and in pilot human trials. Studies in animal models of spontaneous autoimmune diabetes (NOD mouse) have shown that parenteral and nasal administration of GAD65 can prevent (or delay) the onset of the disease. Induction of oral tolerance requires a prolonged administration of autoantigens, in the range of mg/week/mouse.

Poor GAD protein solubility in bacteria and inadequate production from eukaryotic cells have so far

precluded the use of this approach for the large scale production of GAD65 for oral tolerance studies .

Transgenic plants expressing high level of recombinant human GAD65 could be a new and economic source of food for oral administration of the autoantigen. We previously reported the production and characterization of transgenic plants that express membrane-anchored hGAD65 (Porceddu *et al.,* 1999) and the production and characterization of plants expressing a cytosolic form of the recombinant protein (GAD67/65) (Avesani *et al.,* 2003). By using a radio-immuno assay (RIA) with human serum from a GAD65 autoantibody positive T1DM patient, the highest expression level of the recombinant GAD67/65 protein was estimated to be 0.19% of total soluble protein, compared to only 0.04% of hGAD65.

To improve expression levels of recombinant hGAD65 we have used seed-specific expression signals for seed accumulation in different plant species. The sequence encoding GAD67/65 enzymatically inactive (mutated in the active site) was cloned between the N-terminal signal sequence of the seed storage protein 2S2 for ER-targeting and the C-terminal KDEL sequence for ER-retention. This open reading frame was brought under control of the β-phaseolin promoter of *P. vulgaris* with an Ω leader and of the 3' regulatory sequence of the arc5-I genomic clone. This expression cassette was used to transform *N. tabacum* and *A. thaliana* plants. To compare different plant expression systems we also plan to transform *P. hybrida* and *M. truncatula* plants.

Total soluble proteins were extracted from seeds of transgenic tobacco and Arabidopsis plants and Western blot analysis was performed to test the presence of the recombinant hGAD65.The highest expression level of hGAD65 was estimated to be 0.1% of the total soluble proteins.

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Poster Abstract – F.02

PRODUCTION OF AN ANTIMICROBIAL KILLER PEPTIDE IN TRANSGENIC TOBACCO

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killer peptide, antimicrobial, transgenic plants, Agrobacterium tumefaciens

The decapeptide Killer Peptide (KP) derived from the sequence of a single-chain anti-idiotypic antibody, was shown to exert a strong microbicidal activity against both human and plant pathogen. (Polonelli *et al.* 2003, Infect. Immun. 71: 6205-6212; Donini *et al*. 2005, Appl. Env. Microbiol., 71: 6360-6367). It was also shown that the KP expressed in plants by using a Potato Virus X (PVX)-derived vector as a fusion to the viral coat protein (CP), yields chimeric virus particles (CVPs) displaying the heterologous peptide endowed with a potent microbicidal activity. Furthermore in planta challenge assays demonstrated that the KP-CVPs protect Nicotiana benthamiana plants from the bacterial infection of Pseudomonas syringae pv. Tabaci (Donini *et al.* 2005, Appl. Env. Microbiol., 71: 6360-6367).

In this work we focused on the expression of KP in Nicotiana tabacum by using *Agrobacterium tumefaciens* mediated transformation in order to evaluate the protection conferred to a broad spectrum of both bacterial and fungal pathogens. Moreover transgenic plants are evaluated for their potential as biofactories for large-scale production of the microbicide. To this aim different transformation vector cloning strategies of the KP were pursued. We constructed five different KP expression vectors all harbouring the nptII Kan resistance gene, the CaMV 35S promoter and the tobacco mosaic virus (TMV) Ω translational enhancer sequence.

The KP sequence was cloned as a single ORF, however due to the stability issues of this peptide, we also designed a new molecule introducing two identical KP sequences fused by a linker peptide able to form intrachain disulfide bonds. The rationale behind this construct was to stabilize the molecule maintaining its antimicrobial activity. The other constructs were obtained through the N-terminal fusion of the KP peptide to a non functional form of the PVX CP that was used as a carrier to stabilize and enhance the production yields of the peptide. The expression of these constructs was directed either in the cytoplasm or to the secretory pathway by adding a polygalacturonase inhibiting protein (PGIP) signal peptide; moreover a specific restriction site for proteolysis was inserted between the KP and the CP sequence to facilitate the purification process of the peptide. To rapidly evaluate the in planta behaviour of the different gene constructs, we performed transient expression analysis through agroinfiltration of N. tabacum leaves. We evaluated the expression of the different PK constructs through Western Blot analysis and ELISA. N. tabacum plants were stably transformed with the different gene constructs, obtaining 50 independent transgenic lines for each construct. Transgenic plants will be evaluated for both pathogen resistance and expression yield of the heterologous protein.

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Poster Abstract – F.03

EXPRESSION OF HUMAN LECITHIN-CHOLESTEROL ACYLTRANSFERASE (LCAT) GENE INTO TOBACCO PLANTS

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lcat, lecithin:cholesterol acyl transferase, Nicotiana tabacum, HDL, molecular farming

Lecithin: cholesterol acyl transferase (LCAT; EC 2.3.1.43) is a glycoprotein synthesised by liver and secreted into the plasma, where it circulates bound to HDL and LDL complexes. LCAT is responsible for the esterification of free cholesterol and allows formation of mature HDL complex. So, LCAT plays an essential role in reverse cholesterol transport. However, mutations in *lcat* gene cause two human rare forms of dyslipoproteinemia known as familial LCAT deficiency (FLD) and fish eye disease (FED).

In a frame of a Telethon project, in order to study *in vitro* wild and mutated LCAT enzymes to ascertain their physiological activities, it is necessary to have a large amount of those proteins, perhaps expressed in an heterologous system to avoid any interferences with other human or mammals proteins. In this presentation we report the LCAT wild type gene expression into *Nicotiana tabacum* plants. LCAT cDNA was transferred into a pGREEN binary vector, under the control of CAMV 35S promoter, giving rise to the plasmid called pG0029-LCAT. Nuclear genomic DNA amplification by PCR, using specific primer, showed the presence of *lcat* cDNA in 7 of 36 putative transgenic shoots rooted on MS medium added with kanamycin. To confirm the presence of *lcat* transcript, RT-PCR analysis was carried out. This analysis confirmed the presence of *lcat* transcript in 6 of 7 tested plants. Hence, Western analysis was performed to verify the expression of the LCAT enzyme in transformed tobacco plants. Rabbit polyclonal antibody against LCAT protein detected the presence of recombinant LCAT enzyme into transgenic plants. Identification of transgene copy number and quantification of LCAT recombinant enzyme are now in progress.

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Poster Abstract – F.04

SARS-CoV NUCLEOCAPSID AND MEMBRANE PROTEINS EXPRESSED IN PLANTS FOR THE DEVELOPMENT OF LOW COST DIAGNOSIS TEST AND ORAL VACCINE.

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SARS-CoV, plant-derived antigen, PVX

The *Coronavirus* SARS-CoV is the aetiological agent of the Severe Acute Respiratory Syndrome (SARS), which caused a worldwide infectious emergence in 2003. There is no specific therapy or vaccine against this diseases, thus we have started investigations in this area as well as for the generation of low cost diagnostic tests. Working with SARS-CoV is potentially hazardous, as the virus has a recognised ability to infect laboratory workers, and there is a need to provide a safe way to produce SARS antigens that can be manufactured with minimal risk.

The SARS-CoV M and N genes (encoding for the most abundant proteins, respectively, in the virus core and in the viral envelope) were previously reconstituted by RT-PCR on viral RNA extracted by the SARS-CoV Frankfurt isolate and were expressed in *E. coli* to develop a serological assay for diagnosis of SARS. As the integral M protein is toxic in *E. coli* and N protein is mainly expressed in inclusion bodies, we decided to express these proteins in *Nicotiana benthamiana* plants by using a Potato Virus X (PVX)-derived vector, to try to get these proteins in native form. Both proteins were obtained in systemic leaves after PVX infection.

In particular, the N protein was expressed as soluble (about 3 µg recombinant protein/g of fresh plant tissue) and was specifically recognized by rabbit and mouse hyperimmune sera that also recognize the protein in SARS-CoV infected cells. With the aim to evaluate the immunogenicity of the plant-derived antigens and to develop a diagnostic test based on plant extracts containing the SARS-CoV N protein, we are evaluating the reactivity with the plant extracts of sera from patients with confirmed SARS diagnosis.

The results might open the way for the development of low-cost plant-derived SARS-CoV diagnostic tools. Moreover, we are pursuing this approach for the generation of (edible) vaccines against SARS.

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Poster Abstract – F.05

HIV-1 GAG PROTEIN PROCESSING IN TOBACCO CELLS

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HIV-1, gag, tobacco, HIV-1 protease,

The HIV-1 Gag protein (55 KDa) is involved in auto-assembling and formation of VLPs (Virus Like Particles) in human cells. It is composed of four distinct domains, the N-terminal matrix domain (p17), the central capsid domain (p24), the nucleocapsid domain (p7) and the C-terminal domain (p6). Two spacer peptides, p1 and p2, separate the p24/p7 and p7/p6 domains, respectively (Morikawa Y. 2003 Curr. HIV Res. 1: 1-14). In infected human cells, the Gag protein is synthesized as a precursor and converted in the mature protein by the viral protease. We expressed the Gag protein in tobacco cells using two different strategies: stable transformation of the plastidial genome and transient expression in the nucleus via *A. tumefaciens*.

In transplastomic plants, western blot analysis using a recombinant polyclonal HIV/p24 antiserum detected a strong signal of about 40 KDa and weak signals of about 55 and 24 KDa, not present in negative control plants. The 40 KDa product was detected also using a HIV/p17 antiserum. Moreover, when we incubated the protein extracts with a recombinant HIV-1 protease for different times (5-120 min), a rapid reduction of the 40 KDa band and a concomitant increase of the 24 KDa product were observed. These results demonstrate that the 40 KDa product consists of the N-terminal matrix (p17) and the central capsid (p24) domains. Hence, the presence of multiple bands is due to the processing of the Gag polypeptide by plastidial proteases similarly to what occurs with the viral protease.

In nuclear transient expression experiments, we agroinfiltrated tobacco plants (*N. tabacum* and *N. benthamiana*) with a series of *gag* binary constructs under the control of the *RbcS* promoter and 5'UTR, along with specific signal sequences for protein accumulation in different subcellular compartments (cytosol, apoplast, endoplasmic reticulum, chloroplast, and mitochondrion) and *myc*/6xHis tags. Western blot analyses carried out 3 days post infiltration (dpi) with HIV/p24 antisera did not show Gag protein accumulation in any subcellular compartment. On the other hand, RT-PCR analyses indicated that the *gag* gene was transcribed correctly, albeit at various levels with different constructs. To check whether the lack of expression was due to a particular subunit of the Gag polyprotein, we inoculated plants with constructs containing the p17, p24 or Dp17 domains (the latter including all domains except p17). Western analyses, carried out 3 dpi, detected the 24 KDa protein in plants agroinfiltrated with the p24 construct, whereas no signal was observed for the p17 construct. In the Dp17 infiltrated plants, protein analysis with HIV/p24 antiserum revealed only the p24 protein, suggesting that the native protein is quickly processed by plant proteases as already observed in *gag* transplastomic plants.

These results are useful to develop novel vectors and transformation strategies for the expression of the *gag* gene in plant cells.

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Poster Abstract – F.06

TAIL-ANCHORS FROM TYPE IV MEMBRANE PROTEINS AS A TOOL TO INCREASE ACCUMULATION OF AN HIV ANTIGEN

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Tail-anchored, cytochrome b5, Nef - HIV, transgenic plants

Transgenic plants as bioreactors have been used to produce recombinant proteins for medicinal purposes, including mammalian antibodies, blood substitutes and vaccines, because they represent a safe, effective and inexpensive way to produce pharmaceuticals. As the demand for biopharmaceuticals is expected to increase, transgenic plants have the potential to provide virtually unlimited quantities of proteins to be used as tools in both human health care and bioscience.

However, the low cost and highly scalable protein biomanufacturing capacity of plants is often limited by the small amount of the recovered protein. To tackle this limitation we developed a new strategy that was expected to favour the accumulation and the metabolic stability of the recombinant protein in plant tissues. To test our strategy of plant-based antigen production we chose the Nef protein, an HIV antigen that shows promise as a vaccine candidate.

We provided the antigenic Nef molecule with an hydrophobic anchor that allows localization to and concentration of the protein on the cytosolic face of the ER membrane.

The anchoring function is provided by the C-terminal domain of the cyt b(5), a type IV, or tail-anchored (TA), membrane protein. To assay the correct localisation and topology of the fusion protein, we tagged the C-terminus with a sequence containing a consensus for N-glycosylation, which occurs in the ER lumen. Moreover, to purify the Nef protein that lacks the tail-anchor, we inserted a thrombine cleavage site between the C-terminus of Nef and the transmembrane domain region.

Stable transgenic tobacco plants expressing the Nef-TA protein or its soluble cytosolic counterpart (Nef Δ TMD) have been established. Metabolic labelling of transgenic protoplasts indicated that both the anchored and soluble Nef are stable up to 6 hours of chase. Nevertheless, the presence of the tail anchor contributes to an at least 20-fold higher increase in synthesis rate of Nef-TA compared to Nef Δ TMD. The occurrence of N-glycosylation indicates that Nef-TA is inserted into the ER membrane with the correct TA topology.

Intracellular localization of the heterologous proteins in plant cells was investigated using immunofluorescence and confocal microscopy. The distribution pattern of Nef-TA showed a typical ER staining which could be superimposed with that of the microsomal marker BiP.

The ER localization of the anchored Nef has been confirmed by subcellular fractionation experiments.

Our results indicate that the tail anchor from type IV membrane proteins represents a good tool to target recombinant proteins to the ER membrane, thus preventing rapid degradation and/or improving accumulation of the heterologous products.

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Poster Abstract – F.07

EXPRESSING HIV-1 NEF PROTEIN IN TOBACCO PLANTS

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plant vaccines, HIV-1, Nef

The possibility to synthesize biopharmaceuticals (such as vaccine components) using plants paves new ways and offers solutions to some of the problems associated to traditional foreign expression systems (Ma *et al.*, 2005 Vaccine 23: 1814-18).

Modern vaccines are becoming increasingly complex, foreseeing, for example, the incorporation of multiple antigens. Approaches towards developing HIV vaccines appear to confirm this trend, with a combination of candidate antigens being recommended by several groups.

Plant expression of a number of these candidates has already been achieved, including HIV-1 gp120 envelope glycoprotein, p24 core protein and the regulatory Tat protein. Both regulatory and accessory HIV proteins are currently regarded as promising targets for vaccine development as they could provide further protective efficacy in combination with viral structural proteins. In this regard, HIV-1 accessory Nef protein is considered a promising target (Robert-Guroff 2002, DNA Cell Biol. 21: 597-8).

Nef is incorporated into viral particles and expressed in the early stage of infection both in the cytoplasm and on the cell membrane of virus-infected cells. Nef interacts with multiple host factors in order to optimise the cellular environment for virus replication. Its critical role in pathogenesis is demonstrated by the fact that the infection with *nef*-defective HIV strains dramatically decreases the rate of disease progression in seropositive individuals (Tobiume *et al.*, 2002, J.Vir. 76: 5959-65). Moreover, Nef could be an important component for CTL-based HIV-1 vaccines, therefore immune responses directed against this viral protein could help to control the initial steps of viral infection and reduce viral loads and spreading (Robert-Guroff 2002, DNA Cell Biol. 21: 597-8).

Studies of genetic characterization of nef gene showed that two proteins could be translated *in vitro* and expressed in mammalian cells: a full-length N-terminal myristoylated form of 27 kDa (p27) and a truncated form of 25 kDa (p25) lacking the first 18 amino acids (Kaminchik *et al.*, 1991, J Vir. 65: 583-8).

To explore the possibility of plant Nef expression, a number of different constructs have been used to generate independent transgenic lines of *Nicotiana tabacum* cv. Petit Havana. A number of transgenic lines expressing at high levels both p27Mut and p25 Nef isoforms have been identified. Characterization of these transgenic lines is currently being performed.

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Poster Abstract – F.08

EXPRESSION IN PLANTS OF PROTEINS FROM HPV8, A CUTANEOUS HUMAN PAPILLOMAVIRUS

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molecular farming, cutaneous human Papillomavirus, HPV8, transgenic plants, agroinfiltration

Non melanoma skin cancer (NMSC), including basal cell (BCC, 80% of NMSC) and squamous cell carcinoma (SCC, 20%) is the most frequent cancer in Caucasians. Its incidence has dramatically increased in the last years due to sun exposure and immunosuppression: i.e. transplant recipient have a 100-fold and a 10-fold risk increase of developing SCC and BCC, respectively. Sensitive diagnostic techniques have detected a few Human Papillomavirus (HPV) species in lesions of patients affected by these tumours, particularly belonging to the Beta-papillomavirus Genus. This Genus includes viruses with a cutaneous tropism, while high risk HPVs , responsible of almost all cervical cancers in woman, have a mucosal tropism. A few Beta HPVs, such as HPV8 and HPV5, are found associated with skin cancer lesions and are thus considered etiologically related to these pathologies.

For preventing mucosal HPV infection, prophylactic vaccines based on the viral L1 major capsid protein, assembled into empty virion like particles are already available. Preventive vaccines targeting non structural viral proteins, such as the oncogenic proteins E6 or E7 are also under study. Instead, for cutaneous HPVs, no vaccine strategies have yet been described.

Plants have been proposed as alternative platforms for producing foreign antigens for vaccine production and immunisation purposes. Antigens expressed in plants can be administered in different ways, including direct oral ingestion of plant tissue (edible vaccine).

We have recently undertaken a project aimed at obtaining vaccines against cutaneous HPVs, based on the expression in plants of the major L1 capsid and of the E7 protein of HPV 8. To evaluate protein expression in plants tissue, polyclonal antibodies were raised in rabbits against purified GST-L1 and GST–E7 fusion proteins, produced in *E. coli*.

For the expression in plants, 35S-based cassettes for (1) the full length L1 protein, (2) an L1 protein deprived of a putative nuclear localisation signal, (3) the E7 protein and (4) an L1-E7 fusion construct were thus generated and used for both transient and stable expression experiments. For transient expression, infiltration of *N. benthamiana* leaves with *A. tumefaciens* carrying the different constructs (agroinfiltration) was carried out. Transgene expression was followed both at the RNA and protein level. Simultaneous infiltration of agrobacteria carrying genes for silencing suppressors, such as p19 from *Cymbidium ringspot virus*, and/or HC-Pro from *Potato virus Y* enhanced transgene expression of all constructs. Data concerning the molecular characterisation of *N. benthamiana* lines transgenic for the L1 and E7 antigens and their derivatives will be presented.

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Poster Abstract – F.09

EXPRESSION OF HPV L1 GENE IN TOBACCO CHLOROPLASTS

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plant made pharmaceuticals, HPV, plastid transformation

Biopharmaceuticals have traditionally been produced in a variety of transgenic systems. Also plants can be used as a source of therapeutic proteins and are now considered a promising production platform. They have been shown to have several advantages over the conventional systems, such as: lower costs, easy of scalability, absence of health risks. The objective of this project is the expression in tobacco plants of a viral antigen, the L1 protein from the Human Papillomavirus (HPV), a virus which causes the cervical cancer. To date, cervical cancer is the fifth leading cause of death for women worldwide. L1 gene codes for the major capsid protein which forms Virus Like Particles (VLP). VLPs mimic the infection with virions and induce virus-neutralizing antibodies. Thus, VLPs are the most attractive candidate for developing a prophylactic vaccine against HPV infections. To overcome the limit of a low yield of recombinant protein, we used plastid transformation, which allows to deliver the transforming DNA in the plastid genome, rather than the nucleus. Compared to conventional transgenic technologies, plastid engineering generally shows higher protein expression levels, no gene silencing, transgene containment due to the absence of plastids in the pollen.

Several vectors containing the wild-type L1 sequence were constructed, in which L1 is fused to either the GST or the His tag and regulated by strong plastid promoters and different 5'UTRs. In order to test the effect of codon usage and nucleotide sequence on transgene expression, the gene sequence was also changed according to the plastid codon usage and four new vectors carrying the plastid modified sequence were assembled. Spectinomycin resistant transplastomic plants were obtained following biolistic DNA delivery. Correct gene insertion and homoplasmy level were tested by PCR and Southern blot analyses.

RNA gel blot analyses were carried out to verify the presence of L1 transcripts in tobacco plastids. The presence of both monocistronic and dicistronic messages was shown. Western blot analysis carried out on *E. coli* total cellular proteins using an anti-His antibody showed that L1 protein largely accumulates in the insoluble fraction of *E. coli*. By contrast, no protein was detected in plant extracts by western blot using either anti-tag or anti-L1 antibodies. Pulse-chase labeling experiments demonstrated that the protein slightly accumulates in plastids, but it is highly unstable, since the signal decreases after 4 hour chase, almost disappearing after 24 hours.

Supported by recent data found in literature, new vectors with a human-optimized version of the gene are under construction.

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Poster Abstract – F.10

THE DEVELOPMENT OF TRANSGENIC AND TRANSPLASTOMIC PLANTS FOR PRODUCTION OF A TUBERCULOSIS SUBUNIT VACCINE

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mucosal delivery, transgenic plants, LTB, tuberculosis vaccine

Mucosal delivery of a vaccine can induce systemic and mucosal immune responses and eliminate the need for needle and syringes. Transgenic plants are an ideal means to produce mucosal vaccines, as the plant cell wall protects the antigenic proteins from the acidic environment of the stomach. The purpose of this study was to evaluate the potential of an oral, plant-made, tuberculosis vaccine based on the immunodominant antigen ESAT-6 (6-kDa early secretory antigenic target). A synthetic, plant-optimized, coding region was constructed for the antigen ESAT-6. The resulting coding region was fused to the B subunit of the *Escherichia coli* heat-labile enterotoxin (LTB) to promote targeting to the antigen presenting cells beneath the lining of mucosal surfaces. The gene for the LTB/ESAT-6 fusion protein has been expressed in the model plant species *Arabidopsis thaliana* and in *Lycopersicum esculetum* (tomato). Both *A. thaliana* and tomato produced a fully assembled and functional antigen. Preliminary results indicated that, in mice, oral delivery of the plant-made LTB-ESAT-6 fusion protein induced antigen-specific responses from $CD4^+$ cells and IFN-g. In addition, a type 2 response was induced in the Peyer's Patch. Thus, the plant-made antigen was delivered to the gut-associated lymphoid tissue.

In comparison with conventional nuclear transformation, plastid transformation is reported to have significant advantages, such as high gene expression and transgene containment. Hence, in this study, the fusion gene LTB/ESAT-6 was cloned also in plasmids for stable plastid integration and expression. Transplastomic tobacco plants were produced by bombardment of tobacco leaves with the construct containing the fusion gene LTB/ESAT-6. Incorporation of the fusion gene in the plastid genome was confirmed by polymerase chain reaction (PCR) analysis. Western Blot and ELISA analyses are under way to verify transgene expression, product size and estimate antigen expression level.

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Poster Abstract – F.11

HINTS FOR THE SUCCESSFUL DISPLAY OF PEPTIDES ON THE COAT PROTEIN OF POTATO VIRUS X FOR VACCINE FORMULATIONS

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peptide display, PVX CVPs, PVX mutant, plant-virus interaction

A promising application of engineered plant viruses to biopharmaceutical research is aimed to the development of peptide vaccines, utilising chimeric virus particles (CVPs) displaying on their surface, as fusion to the coat protein (CP), peptides of immunological interest (epitopes). The purified CVPs can then be used as carriers for the delivery of immunogenic peptides that can induce speific antibody responses while harmless for human/animal health.

In this work we have exploited a spontaneous Potato Virus X (PVX) mutant that express a truncated, but functional, form of the CP lacking 20 residues (2 to 22) at the N-terminus. Two pPVX*201*-derived viral expression vectors encoding the *cp* mutant gene have been constructed to fuse, to the 5'-terminus of the *cp* gene, the sequences encoding a panel of peptides (26 in total) varying both in length and amino acid composition. The ability of each chimeric CP to enable virus spreading was evaluated investigating the infection progression induced by each construct *in planta*. From this analysis we were able to define that Tryptophan content and isoelectric point, are two fundamental structural features of the fused peptides that influence CP functions. Depending on the amino acid composition, fusion peptides are able to critically affect both local or systemic movement of PVX. Moreover, we evidenced that the genetic stability and preservation of the correct peptide sequence is heavily influenced by the percent content in serine and threonine of the fused peptide.

Our findings, while describing the effect of additional peptides on virus infectivity (movement), provide new insights into the prediction of peptide sequences that are compatible with the display on PVX and the most favourable conditions to generate infectious CVPs to produce candidate vaccines.

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Poster Abstract – F.12

RISK AND STABILITY ASSESSMENT OF A POTATO VIRUS X-BASED VECTOR FOR RECOMBINANT PROTEIN EXPRESSION

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potato virus X, transient expression, recombinant protein, risk assessment, viral recombination

We investigated the stability of expression constructs based on Potato Virus X (PVX) as a function of insert length. Five different inserts ranging in length from 261 to 1758 bp (human proinsulin, murine interleukin-10, HIV-1 *nef*, petunia expansin-1 and human *GAD65*) were expressed using a PVX vector in *Nicotiana benthamiana* plants for three sequential passages. Using a competitive RT-PCR approach we demonstrated that all five inserts could be deleted in the first infection cycle, but that this was much more likely to occur for longer inserts. This suggested a negative correlation between insert length and vector stability. Sequence analysis of the deleted constructs suggested that recombination usually occurred at sites close to the duplicated subgenomic promoter, but in a smaller number of cases the foreign gene itself was probably involved resulting in partially deleted constructs containing transgene fragments. The implications of these results in the context of viral replication and the risk assessment of expression vectors based on plant viruses are discussed.

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Poster Abstract – F.13

CHEMICAL CHARACTERIZATION OF SAPONINS FROM A MUTANT PLANT OF *M. TRUNCATULA*

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Medicago truncatula, saponins, chemical characterization

In a collection of T1 transgenic plants of *M. truncatula* obtained by inserction mutagenesis (activation tagging procedure) and screened for the presence of haemolytic saponins, one plant showing no haemolytic activity was evidenced. The T2 progeny (30 plants) was grown to investigate the transmission of the phenotype and to determine the number of insertion fragments by Southern analysis.

Preliminary investigations of saponins and sapogenins of this mutant plant showed a completely different composition compared to the untransformed parental strain used as control. Saponins were than extracted in water-methanol mixture, purified by reverse-phase chromatography and analyzed by different chemical methods to obtain information on their structure.

Structure investigation of saponins was performed by identification of sapogenins and sugars released after acid hydrolysis from pure saponins. Detailed information on the saponin structure was obtained by a combination of analytical methods including HPLC, NMR, MS analyses performed on the pure compounds.

The differences in qualitative and quantitative composition allowed to obtain information on the biosynthetic pathways of the aglycone moyeties, involving the squalene-2,3-epoxide that, after cyclization to give the corresponding pentacyclic triterpene nucleus, gives the different sapogenins by successive oxidation steps.

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Poster Abstract – F.14

TOWARDS PRODUCTION OF SAPONINS IN CALLUS AND CELL CULTURES IN *SOLANUM* AND *ASTER*

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Solanum lycopersicum, Aster sedifolius, Aster caucasicus

Plants are able to synthesize a wide range of molecules potentially useful to control phytopathogenic organisms, such as virus, bacteria, fungi and insects. The use of natural compounds to control biotic agents in agriculture is of much interest for safety and ecological reasons. Species belonging to the genus *Solanum* (fam. *Solanaceae*) and *Aster* (fam. *Asteraceae*) produce high levels of steroidal and triterpenoid saponins respectively, that show biological activities. The synthesis of these molecules in plants is dependent on environmental conditions or a particular growth stage of the plant. Plant cell culture is an alternative and renewable source of secondary metabolites and could supply the production of, bioactive secondary metabolites on industrial scale. The main goal of this study is to obtain production of saponins from callus and cell cultures of *S. lycopersicum*, *A. sedifolius* and *A. caucasicus.* In order to obtain *in vitro* cultures of the three species here reported, we started to set up optimal conditions for callus and cell production. Three types of explants such as leaf, petiole and root were assayed in Murashige and Skoog solid medium containing vitamins and supplemented with sucrose and with several different combinations of hormones 2,4-dichlorophenoxy acetic acid (2,4-D) and 6-benzylaminopurine (BAP). Leaves were the best source of explants for obtaining a callus culture in *S. lycopersicum* on MS medium supplemented with 2,4D plus BAP. Furthermore, based on weight increase, leaf explants performed better under the light than under the darkness. In *A. caucasicus*, high callus production was successfully induced only in petiole explants collected from *in vivo* plants. Callus growth was greater on MS supplemented with 2,4D than in other tested media and it was not affected by light. Conversely, in *A. sedifolius* leaf explants from plants grown *in vivo* produce callus on MS medium supplemented with 2,4-D and BAP. As concerning callus induced under the light, a large variability has been detected for colour and types (compact or friable) of the calli. Cell cultures were established by subculturing friable calli for each species into the optimal medium tested for callus production.

Calli and cells in different growth stages, exponential growth phase, linear phase and stationary phase will be assessed by molecular and biochemical analysis to evaluate the production of saponins.

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Poster Abstract – F.15

ESTABLISHMENT OF IN VITRO CELL CULTURES OF *ARTEMISIA ANNUA* L. FOR THE PRODUCTION OF THE ANTIMALARIAN PHYTOCHEMICAL ARTEMISININ

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Artemisia annua L., cell culture, secondary metabolite production, antimalarian phytochemical, artemisinin

Artemisia annua L. is an aromatic annual herb which has been used in Chinese medicine for centuries in the treatment of fevers. However, the plant now grows wild in many other countries in Europe and America. There is a great interest in this plant due to its ability to synthesize and accumulate a variety of secondary metabolites which are biologically active compounds. Among them, artemisinin, an endoperoxide sesquiterpene lactone, is an antimalarian drug effective against multidrug resistant strains of *Plasmodium,* the malarial parasite. At present, the major production of artemisinin for pharmacological use is obtained by extraction and purification from field grown plants, although with quite variable yields. The highest artemisinin content has been found in leaves and flowering buds of the plant, and it is influenced by several environmental factors. As a result, the supply of artemisinin is far from enough in the international market. On the other hand, the chemical synthesis has not proved to be commercially feasible. Efforts are needed in order to enhance the production of artemisinin in plants or, alternatively, to exploit cell and tissue culture technology, which has not been fully explored in this species.

The aim of this work is the establishment of cell and tissue cultures of *Artemisia annua* L. for the *in vitro* production of artemisinin.

Axenic leaf discs were incubated in the dark on various media in order to induce callus cultures. A broad spectrum experimental design using MS basal medium supplemented with 49 combinations of NAA or 2,4D with 6-BAP at different concentrations was carried out. Quantitative and qualitative evaluations of callus proliferation were performed in several subcultures allowing to identify the most suitable conditions for the establishment of cell suspensions. The best results were obtained when 2,4D and 6-BAP were added in the ratio of 5:1 to MS basal medium.

Starting from axenic meristem tips it was also possible to obtain shoot cultures which were micropropagated on MS basal medium supplemented with 6-BAP (0.5 mg/L) and NAA (0.01 mg/L). This result was obtained when the optimal medium composition able to reduce the occurrence of vitrification was identified, by lowering auxin and increasing agar concentration.

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Poster Abstract – F.16

MEMBRANE INSERTION AND N-GLYCOSYLATION OF VACCINIA VIRUS A33R PROTEIN IN TOBACCO PROTOPLASTS

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molecular farming, Vaccinia virus, membrane proteins, tobacco protoplasts

Molecular farming in plants has many practical, economic and safety advantages compared with more conventional systems, and plants are becoming an attractive system for large-scale recombinant protein production. We are investigating the possibility to produce in plants antigens of Vaccinia virus, the orthopoxvirus used for vaccination in the worldwide eradication of smallpox .

Investigation on Vaccinia virus showed that the interference with the expression of any protein present in purified extracellular enveloped virus (EEV) by deletion or repression has dramatic impact on the ability of the virus to spread from cell to cell in tissue culture and form plaques. Further evidence attesting to the importance of the EEV comes from antibody studies. Several researchers have reported that antibodies to EEV proteins protect animals from viral challenge significantly more than antibodies to the intracellular mature virion.

A33R is one of EEV specific proteins and it is a highly conserved among other orthopoxviruses. It is a 23-28kDa N- and O-glycosylated type II integral membrane protein, with a short N-terminal cytosolic domain. In this study we report the transient expression of the A33R protein in protoplast of tobacco. To this aim we cloned in the vector pDHA the A33R DNA coding sequence, fused to a tag of eight amino acids at the C-terminus (FLAG epitope). Radioactive A33R can be immunoprecipitated with anti-FLAG antiserum and is still detectable after four hours of chase in a pulse-chase experiment. The protein is N-glycosylated, as indicated by a reduction in molecular mass upon pulse-chase performed in the presence of the N-glycosylation inhibitor tunicamycin. N-glycosylation indicates correct insertion into the endoplasmic reticulum membrane. Protein blot analysis suggests that the protein can form dimers (around 55kDa), similarly to what happens in animal cells.

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Poster Abstract – F.17

A γ-ZEIN-NEF FUSION IS UNSTABLE IN TRANSGENIC TOBACCO LEAVES

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human immunodeficiency virus, zein, protein secretory pathway, tobacco

Plants are being investigated as a possibly safer and less expensive alternative to microorganisms, animal cell cultures or transgenic animals for the production of recombinant pharmaceuticals. In this respect, production of antibodies has proved to be qualitatively and quantitatively efficient, whereas recombinant antigen accumulation has often been unsatisfactory. We are trying to produce high amounts of recombinant Nef, a human immunodeficiency virus antigen, in transgenic tobacco by taking advantage of the high stability of proteins that form protein bodies within the endoplasmic reticulum of plant cells. Nef is a cytosolic protein that has been up to now recalcitrant to high accumulation in plants. We have previously shown that an N-terminal domain of the maize seed storage protein g-zein fused to the vacuolar plant protein phaseolin highly enhances the accumulation of the latter expressed in leaves of transgenic plants, most probably because the chimeric protein (termed zeolin) forms very stable protein bodies within the endoplasmic reticulum, thus avoiding degradation in vacuoles or other compartments of the secretory pathway. A similar γ-zein-Nef fusion was constructed and expressed in transgenic tobacco. In spite of the high accumulation of mRNA, γ-zein-Nef protein accumulates at low levels. Pulse-chase analysis indicates that the protein has a half-life of less than 2 hours and is degraded in a process that cannot be blocked by brefeldin A, an inhibitor of traffic along the secretory pathway. These results suggest that the fusion protein, unlike zeolin, may be recognized as a structurally defective protein by the endoplasmic reticulum quality control mechanisms. These results indicate that the ability of the zein domain to promote stable protein body formation is influenced by the target protein used to produce the fusion. The structural features that determine the different destinies of zeolin and γ-zein-Nef are under investigation. This can increase our knowledge on the regulation of protein quality control in plant cells and help in the planning of further, possibly more stable, constructs.

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Poster Abstract – F.18

LOCALIZATION OF THE RECOMBINANT PROTEIN ZEOLIN IN THE CHLOROPLAST

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zein, phaseolin, chloroplast, BiP, heterologous protein accumulation

Plants are potentially useful for the production of many therapeutic proteins and several strategies have been exploited to maximize heterologous protein accumulation in the plant cell. Recently, a fusion protein made between the bean storage protein phaseolin and the N-terminal portion of maize prolamin storage protein γ-zein was shown to accumulate in novel ER-derived protein bodies of vegetative tissues, while γ-zein and phaseolin usually accumulates in the ER and in storage vacuoles respectively. The γ-zein fragment, on the basis of the knowledge acquired on zeolin, could be exploited for the production of large amounts of recombinant pharmaceuticals in transgenic plants. In this study we investigate if zeolin could be also expressed in the chloroplast and not only in the ER. In fact, together with others numerous advantages such as high-level of transgene expression, in many crop species chloroplast DNA is not transmitted through pollen thus eliminating the risk of transgene spreading in not transgenic crops or relative wild species. The *zeolin* gene has been cloned in three plastid expression cassettes. Two of them have been assembled to evaluate the utility of including in the zeolin gene the region that encodes the signal peptide. In the third construct the *BiP* gene, which encodes an ER chaperone, has been cloned downstream of the zeolin gene. The presence of the signal peptide seems to promote zeolin accumulation in the chloroplast, even though a comparison between the two zeolin sub-cellular localizations shows that zeolin has accumulated in the chloroplast to lower levels than in the ER. Immunoblotting of the transplastomic plant protein shows the considerable presence of phaseolin and γ-zein polypeptides with respect to intact zeolin. This suggests that in the chloroplast a higher amount of zeolin is fragmented in comparison to zeolin accumulated in the ER. Moreover, no protein bodies have been detected in the chloroplast. In order to promote zeolin folding into protein bodies in this organelle, we have integrated the *BiP* gene together with the *zeolin* gene in the tobacco plastome but even in this case transplastomic plants has not accumulated zeolin in protein bodies. We hypothesize that the disulfide bonds necessary for zeolin assembly have not been correctly formed in the chloroplast. This can have caused zeolin misfolding and not-stable proteins that reach poor expression levels due to chloroplast proteases activity.

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Poster Abstract – F.19

TRANSFORMATION OF BARREL MEDIC (*MEDICAGO TRUNCATULA* L.) WITH THE *OXA1* cDNA FROM *ASTER SEDIFOLIUS*: TOWARDS THE GENETIC MANIPULATION OF TRITERPENE SAPONIN BIOSYNTHESIS

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barrel medic, genetic transformation, triterpenoid saponins

Triterpene glycoside saponins have a range of different properties, including antimicrobial, insecticidal and allelopathic activity. They also have pharmacological applications such as anticholesterolemic, hemolytic, adjuvant and anticancer agents. Objective of this work is to evaluate the possibility to modify the triterpenoid saponin content in *Medicago* spp. by genetic engineering. Flower explants of *Medicago truncatula* (genotype R108) were co-cultivated with EHA105 disarmed *A. tumefaciens* strain. The cloning vector pG0029OXA1 contained the *OXA1* cDNA from *Aster sedifolius* under the control of the cauliflower mosaic 35S promoter, and the neomycin phosphotransferase II (*nptII*) gene. *OXA1* cDNA encodes for a β-amyrin synthase, a key enzyme involved in biosynthesis of triterpene glycosides. After callus induction and *in vitro* embryogenesis, putative transgenic plantlets were regenerated and then micropropagated for further analyses. The presence of *nptII* and *OXA1* genes was demonstrated by PCR analysis. Molecular and biochemical characterizations are currently underway.

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Poster Abstract – F.20

PERSPECTIVES OF PRODUCING BIOPESTICIDES THROUGH OVER-EXPRESSION OF A TRITERPENE CYCLASE GENE

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Aster, metabolic engineering, Agrobacterium transformation

Environmental contamination caused by synthetic pesticides is a well known problem in agriculture. An alternative pest control can be managed by replacing synthetic pesticides with biopesticides, compounds of natural origin, many of which produced by plants against pests and diseases. In *Asteraceae*, a rich chemical diversity exists to such an extent that the evolutionary success of the family was suggested to be due to the highly diversified chemical defence system. In particular, triterpenoid saponins, abundant in this family, have an important role for their activity against fungi, bacteria, insects and nematodes.

A phytochemical analysis of leaves from *Aster sedifolius* led to the isolation of three novel triterpenoid saponins which showed inhibitory activity against different pathogenic fungi, *Rhizoctonia solani, Fusarium solani, Sclerotium rolfsii* and *Sclerotinia spp.*, as well as against a bacterium, *Xanthomonas campestris*. In this context, our research group isolated in *A. sedifolius OXA1* gene encoding a key enzyme of the triterpene pathway performing cyclization of 2,3 oxidosqualene into pentacyclic carbon skeleton. To increase the production of triterpenoid saponins, a metabolic engineering strategy based on the over-expression of *OXA1* was designed. An expression vector useful for transformation by *Agrobacterium* was constructed with *OXA1* under the control of the constitutive promoter CaMV35S. Strains of *A. tumefaciens* (LBA4404 and EHA105) with CaMV35S::*OXA1* have been utilized to transform different species: *Aster sedifolius* and *A. caucasicus,* as well as *Arabidopsis thaliana* and *Nicotiana tabacum* as model plants. Moreover, *A. rhizogenes* was used for *A. sedifolius* transformation. It is expected that *OXA1* could increase the production of bioactive compounds in *Aster,* and particularly in *A. caucasicus* (species close to *A. sedifolius*) that spontaneously produces saponins inhibiting the growth of pathogenic fungus *Botrytis cinerea*. In *A. thaliana* saponins are absent, but it is known that a multifunctional lupeol synthase gene is expressed and lupeol as well as small amounts of β-amyrin are produced. For this reason, a modification in terpene pathway products could be expected following *OXA1* expression. The results obtained from transformation experiments will be discussed.