‘CLEAN’ STRATEGIES FOR EXOGENOUS GENE TRANSFER IN GRAPEVINE

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In grapevine, the gene transfer technique is based mainly on *in vitro* co-cultures of plant tissues with *Agrobacterium tumefaciens*. The selection of transgenic cells is one of the crucial steps of the overall strategy. Established selection techniques are usually based on resistance genes, that confer to the cells the ability to survive on media supplemented with antibiotic. Thus, their function is limited to the selection phase and their further presence in the plants may be undesirable. Moreover, their use is becoming controversial (regulation 2001/18/CE).

We are exploiting some strategies for transferring the exogenous genes into grapevine plants, and we are focusing on (i) the elimination of the antibiotic resistance genes after the selection step and (ii) the use of alternative marker genes.

The elimination of the marker gene is based on the pX6 vector (provided by The Rockefeller University - N.Y., Prof. Nam-Hai Chua) that contains the *gfp* gene, the cre/loxP recombination system induced with β-estradiol for the site-specific excision of the *nptII* gene (Zuo J. *et al*., 2001, *Nat. Biotech.*, 19: 157-161). In a second construct, the *gfp* gene was replaced with a sequence of Grapevine Virus A (GVA) coat protein in sense and antisense orientation, resulting in a hairpin RNA (pX6-pKcpGVA, Turturo *et al*., 2003, Proc. 14th ICVG Conference, Locorotondo, Italy, 12-17 Sept.). Successful marker gene removal leads to the expression of the reporter gene (*gfp*) or the GVA sequence respectively. Brachetto and Chardonnay embryogenic calli were co-cultured with *Agrobacterium* alternatively carrying one of these two constructs, putatively transgenic cultures were selected on kanamycin and individual somatic embryos were converted into plantlets. Several tests were performed to optimise the induction with β-estradiol. Best results were obtained with the application for one month of 10 mM β-estradiol on buds before stimulating plant elongation. Southern Blot analyses proved transgene integration and the *nptII* gene removal was quantified with Real-time PCR. Efficiency of *nptII* gene excision was observed to vary along the plant, decreasing from the roots to the apical tissues.

Plant cells expressing the *manA* gene from *Agrobacterium* can be made capable of metabolising mannose, otherwise toxic, as an alternative to sucrose. This strategy produced controversial results in grapes (Kieffer *et al*., 2004 *Vitis* 43:35-39). For this reason, extended preliminary assays were necessary to fully understand the effect of mannose on the cultures, before starting the gene transfer experiments. Wild-type embryogenic calli of ‘Brachetto’, ‘Chardonnay’ and ‘110 Richter’ were grown and subcultured monthly on media added with mannose or sucrose or free of the carbohydrate source. No growth and a gradual death was observed in the carbohydrate-free media, while calli cultured on mannose showed no clear evidence of a fatal damage even after a long time (14 months). Similar results were obtained on mature somatic embryos induced to plantlet
conversion. Brachetto, Chardonnay and 110 Richter embryogenic calli were co-cultured with *Agrobacterium* carrying the pNOV2819 construct (courtesy of Syngenta, licensed) containing the *manA* gene. The maintenance of calli and the induction of plant recovery from somatic embryos were performed on mannose-containing media, and molecular analysis are in progress for assessing and quantifying the presence of the exogene insertion. (Thanks to the Autonomous Province of Trento, Project *EcoGenEtic.Com.* )
DETECTION OF TRANSGENE COPY NUMBER IN DURUM WHEAT TRANSGENIC LINES USING REAL-TIME PCR

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durum wheat, transformation, glutenin subunits, real time PCR

Genetic transformation has played a key role in gaining and applying knowledge of the roles of HMW-GS in wheat end-use properties. Reliable and stable expression of transgenes as well as the characterization and field adaptation of transgenic lines are prerequisites for the successful application of gene technology.

Loci that appear to be stably expressed initially can become progressively silenced over generations. The stability and the behaviour of transgenes are influenced by several factors, such as chromosomal location, transgene copy number and their interaction with the host genotype.

Traditionally such factors are characterized using Southern analysis which can be time consuming and laborious. Recent results obtained in various crops indicate that Real-Time PCR could be a powerful tool for the detection and characterization of transgene locus structures. The determination of transgenic locus number through Real-Time PCR overcomes the problems linked to phenotypic segregation analysis and can analyze hundreds of samples in a day making it an efficient method for estimating copy number integrated in a transgenic line.

This study was conducted to determine transgene copy number in transgenic lines and to investigate potential differences in sensitivity, resolution and variability between two different Real-Time chemistries (SYBR Green dye and TaqMan probes). We have applied Real-Time PCR to a set of four transgenic durum wheat lines previously obtained. A total of six experiments (two experiments for each gene) were conducted and standard curves were obtained from serial dilutions of the plasmids containing the genes of interest. The correlation coefficients of the standard curves were rather good, being in the range between 0.95 and 0.97. By using TaqMan quantitative Real-time PCR we were able to achieve estimates of 1 to 42 copies of transgenes per haploid genome in T4 homozygous transformants. Conversely, SYBR Green dye method revealed unable to accurately quantify transgene copy number as it failed in detecting the inserted genes when integrated in few copies.

In our study we assessed Real-Time PCR as a fast, sensitive and reliable method for the detection of transgene copies in durum wheat, which can be a valid alternative to Southern analysis.
CHLOROPLAST TRANSFORMATION IN SUGAR BEET BY PARTICLE BOMBARDMENT

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aminoglicoside 3’-adenylyltransferase (aadA), biolistic apparatus, chloroplast DNA transformation, green fluorescent protein, spectinomycin

In the past twenty years, plastids have become attractive targets in the field of plant biotechnology. Plastid transformation is routinely used only in tobacco, thus it is very important for the application of chloroplast engineering to extend the range of species in which this technology can be achieved. Here, we describe the development of a chloroplast transformation system for the sugar beet (Beta vulgaris L. ssp. vulgaris, Sugar Beet Group) by biolistic bombardment of leaf petioles and the production of sugar beet transplastomic plants. Despite being a dicotyledonous plant and the considerable attention it has received due to its importance as an agricultural crop for sugar production in the Northern Hemisphere, sugar beet is considered to be a recalcitrant species with respect to genetic transformation, displaying poor reproducibility and genotype dependency. Moreover, there are no reports of sugar beet chloroplast DNA transformation at present, making this field of research open to exploration. The production of transplastomic sugar beet lines with maternal inheritance of transgenes could solve problems related to outcrossing between genetic modified varieties and conventional varieties or wild relatives. It is well documented that the sugar beet easily inter-crosses with its wild relative, the sea beet [(Beta vulgaris L. ssp. maritima (L.) Arcang.], or with annual weed beets that occur in the field. Although Beta vulgaris is a biennial crop that is harvested before its reproductive stage and is not expected to flower in the first year, early flowering can occur through occasional vernalization at low spring temperatures. Therefore, unintentional pollination of sea beets or weed beets is possible. The use of chloroplast genetic engineering could drastically reduce the probability of transgenic pollen dispersal.

To develop a chloroplast transformation system for this species, different methods for plant regeneration were tested for all the varieties analysed. Homoplasmic plastid-transformed plants of breeding line Z025 were obtained. Transformation was achieved using a vector that targets genes to the rnl16/rps12 intergenic region of the sugar beet plastome, employing the aadA gene as a selectable marker against spectinomycin and the gfp gene for visual screening of plastid transformants. gfp gene transcription and protein expression were shown in transplastomic plants. Detection of GFP in Comassie blue-stained gels suggested high GFP levels. Microscopy revealed GFP fluorescence within the chloroplasts. Our results demonstrate the feasibility of engineering the sugar beet chloroplast genome.
IMPROVEMENT OF PLASTID TRANSFORMATION IN POTATO (SOLANUM TUBEROSUM SSP. TUBEROSUM)

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plastid transformation, gene expression, Solanum tuberosum

The integration and expression of transgenes in the plastidial genome of higher plants presents several interesting features. In previous reports, using tobacco vectors, plastid transformation in potato ranged from one transplastomic shoot every 15 biolistic shots to one every 35, transformation efficiencies varying with the genotype and the targeting region. Such low efficiencies prevented the use of plastid transformation in more genotypes and for more aims. We improved the plastid transformation efficiency in the important potato cv. Désirée up to one transplastomic shoot every eight shots by selecting resistant calli on spectinomycin containing medium and regenerating shoots on an optimized series of media. That efficiency was further increased to about one event per bombarded plate by designing a set of new transformation vectors carrying homologous potato flanking sequences. In order to increase transgene expression, especially in non-green plastids, we analyzed GFP expression at the transcript and protein level in leaves and tubers of transplastomic plants produced with a number of vectors containing different promoters and 5'-UTRs. Correct integration of the transgene was proved by PCR and Southern blot analyses, while levels of transcript and protein accumulation were determined by northern and western blot hybridisation, respectively. Detectable transcripts accumulated with all vectors and in both organs, although differences between vectors and generally lower expression in tubers than in leaves were observed. The highest transcript accumulation was obtained with the rrn promoter. Further, the GFP protein could be detected in tubers only when this promoter and a synthetic 5'-UTR containing the rbcL ribosome-binding site were used. The results presented are of particular interest for the production of transplastomic potato plants and for developing a system for protein accumulation in potato tubers.
ANALYSIS OF STABLE TRANSFORMED GRAPE PLANTS EXPRESSING GFP TARGETED TO MITOCHONDRIA

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Vitis vinifera, somatic embryogenesis, transformation

The grape genome is now available to the scientific community. In the near future efficient systems for functional analysis of genes will be very useful. For this reason, we developed a protocol able to transform some grape varieties. Two embryogenic cell lines of Vitis vinifera, variety Moscato giallo and variety Frappato, were obtained and used in transformation experiments with Agrobacterium tumefaciens harbouring GFP constructs targeted to mitochondria. Four hundred milligrams of globular embryo stages were treated with Agrobacterium. Four months later, the percent of transformation of Moscato Giallo and Frappato, measured on 100 isolated torpedo secondary embryos, was 60% for Frappato and 100% for Moscato giallo. Three months later, adult plants were analysed at the level of leaves and roots. From transformed leaves, a stable transformed cell line was produced and mitochondria easily visualised. These stable transformed plants and cells will be used to follow alterations of mitochondria during plant development and growth and in response to biotic and abiotic stresses.
ESTABLISHMENT OF TRANSIENT TRANSFORMATION SYSTEMS IN GRAPE LEAVES FOR FUNCTIONAL GENOMICS STUDIES THROUGH GENE SILENCING


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transient transformation, gene silencing, Agrobacterium tumefaciens, particle bombardment

Transient transformation of plant leaves represent a useful tool allowing to study gene function in different plant tissues and it is a time-saving procedure especially for species with long generation times avoiding stable transformation. In this work two methods for grape leaves transient transformation have been tested: the use of Agrobacterium tumefaciens, which can penetrate into outer leaf tissues, and particle bombardment, useful for: Agrobacterium recalcitrant genotypes, different kind of tissues or substrates and when leaves epidermal cells transformation only is required. To establish a method with A. tumefaciens, PDS (phytoene desaturase, gene coding for an enzyme involved in carotenoid biosynthesis) sequence, integrated in a Gateway® binary vector for gene silencing called pK7GWIWG2D, has been used. Underside leaves have been agroinfiltrated by a syringe without needle and analyzed 6 and 12 days after infection. Presence of chlorotic spots due to photobleaching, a PDS silencing symptom, has been found into agroinfiltrated sites. PDS gene silencing has been confirmed by semiquantitative RT-PCR of the corresponding gene transcripts into transformed tissues.

As a second possibility, we are setting particle bombardment method for transient transformation using GUS (β-glucoronidase) as gene marker. Different kinds of shooting parameters and pre and post-bombardment treatments of plant tissues have been tested. Leaf cells expressing GUS have been obtained by these experiments. Further implementation to the method are in progress in order to obtain a good level of epidermal cells transient transformation.

In the near future, establishment of these two transformation methods will allow use them, in association with gene silencing system, as tools for functional genomic in order to study gene function in different leaf tissues and, in particular, to study genes involved in plant response to biotic stress.
MUTAGENESIS OF MEDICAGO SATIVA GSA-AT FOR GABACULINE RESISTANCE

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Selectable marker genes (SMG) of bacterial origin, especially antibiotic resistance genes, represent a valuable tool for plant engineering. However, the use of these SMG has raised concern about the safety of their use in agriculture. Several approaches are now available to eliminate or replace antibiotic resistance genes from transgenic plants. Plant-derived SMG could be a valid alternative to overcome these problems but there are few available. We have identified a possible SMG candidate in the Medicago sativa glutamate 1-semialdehyde aminotransferase (GSA-AT) gene.

In plants, algae and cyanobacteria, the enzyme GSA-AT catalyses the conversion of glutamate-1-semialdehyde into aminolevulinic acid, a step in the synthesis of tetrapyrrole compounds including chlorophyll. This enzyme is irreversibly inhibited by gabaculine (3-amino-2,3-dihydrobenzoic acid). A mutant form of the hemL gene of Synechococcus elongatus encoding GSA-AT has been demonstrated to work well as SMG in tobacco (Gough et al. 2001) and particularly in alfalfa (Medicago sativa L.) (Rosellini et al. 2007).

We found that plant GSA-AT genes have high similarity (more than 70% at the aminoacid level) with hemL: the mutant bacterial protein differs from the wild type for a 3-aminoacid deletion close to the amino terminus and a point mutation resulting in a methionine to isoleucine substitution in the catalytic domain.

We are implementing two strategies of site–specific mutagenesis to induce gabaculine resistance in alfalfa GSA-AT. The first consists in the production of two variants of the alfalfa cDNA: 1) Substitution of the methionine 286 with a isoleucine in the catalytic domain; 2) The same substitution plus the replacement of the aminoterminal of the alfalfa protein with that of the hemL protein.

The second strategy aims to exploit the natural competence and the extremely efficient recombination system of Synechococcus elongatus PCC7942 in order to produce a recombinant strain in which the chromosomal gene hemL is replaced by the cDNA sequence of the alfalfa GSA-AT gene. This recombinant strain will be placed under selection with increasing concentration of gabaculine in the attempt to isolate a mutated, insensitive form of alfalfa GSA-AT (collaboration with the University of Leicester – UK, ADAS group – Garry Whitelam’s lab).
GENE FLOW FROM TRANSGENIC TO RED RICE IN FIELD

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rice, transgenic, herbicide resistance, gene flow, red rice

Outcrossing in cultivated rice (*Oryza sativa* L. ssp. japonica), due to its reproductive biology, is usually considered negligible even if with low frequency it happens. However concerns of environmental risks arise from the possibility of flow of foreign genes inserted in cultivated varieties by genetic engineering (herbicide and parasite resistances), through pollen dispersal to other cultivars of the same species or to weed relatives as red rice (*Oryza* spp.). In particular, that would make more and more difficult the control of this weed with the use of the appropriate chemical principles. Aim of this study was the examine during several years the evolution of the red rice population following its cohabitation with a transgenic line bearing a resistance gene toward ammonium glufosinate. In the experiment the conditions adopted in herbicide transgenic rice cultivations, currently treated with the correspondent herbicide, were considered and reproduced in a crop where the red rice was interspersed with a high but not uncommon frequency: to our knowledge this is the first study performed in such situation. The evolution of the red rice population was examined during a period of five years (2001-2005) in a field cultivated in the first two years with the transgenic line A2504 derived from cultivar Ariete and bearing the resistance to the herbicide. During this period, the field was annually treated with the herbicide at the right time; in the first year the dosage of herbicide was three times higher that the producer (Basta) indication. Nevertheless a plant bearing several characteristics of the red rice was identified, in the experimental field, during 2002. The progeny of several subsequent generations were examined, molecular analysis and herbicide resistance test were carried out and the presence of the transgene was detected, demonstrating the occurrence of the outcross between A2504 and red rice. That shows that a) transgene flow to red rice can occur, b) the transgene remains stable at least for several generations, c) it is actively expressed as a dominant factor in the hybrid and in the following generations.

As a conclusion, from the crops of the transgenic rice, the appearance of red rice progeny tolerant to the herbicide is expected and, consequently, the problem of controlling the weed by chemicals will newly emerge in a relatively short time.
**AGROBACTERIUM-MEDIATED TRANSFORMATION IN DURUM WHEAT**


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**durum wheat transformation, Agrobacteria, cultivar susceptibility**

Durum wheat (*Triticum turgidum* L.) is an important cereal widely used for making pasta, typical bread and semolina. In recent years for many cereals, including durum wheat, biolistic transformation and direct gene transfer methods have allowed the introduction of useful agronomical traits. For the most important cereals, such as rice, maize, bread wheat, sorghum and barley is now possible to provide of an alternative system for genetic transformation based on the activity of *Agrobacterium tumefaciens* (Smith and Towsend) Conn. This mediated transformation has the great advantages over biolistic method to obtain an exact integration of few gene copies into the host genome, and a better stability of the transgene. Many variables can affect the *Agrobacterium*-mediated system, like *Agrobacterium* strain, the natural attitude of plant genotype, the co-cultivation conditions, and the selectable markers.

In this work we report our results concerning the establishment and optimization of a protocol for *Agrobacterium* mediated transformation of durum wheat. We tested different *in vitro* conditions on eight cultivars: Ancomarzio, Bronte, Duetto, Karalis, Neolatino, Ghibli, Sorrento and Vesuvio. The *Agrobacterium* strain AGL1, in different conditions of co-cultivation on 150 immature embryos for each cultivar, was used.

The activity of gusA gene was observed to assess the susceptibility of durum wheat cultivars to *Agrobacterium* infection. The qualitative histochemical assay of transient gusA gene expression, provides useful information on the efficiency of *Agrobacterium* mediated transformation.

The cultivars Vesuvio, Ghibli and Duetto showed many dark blue spots after ten day of *in vitro* culture. The cultivars Ancomarzio, Karalis Neolatino and Sorrento showed different reactions to the histochemical assay, depending on the auxin source used in the experiments, while the cultivar Bronte has always showed low level of coloration.

The cultivars Karalis and Neolatino showed a good level of regeneration and some T₀ plants were obtained. Four Karalis and two Neolatino T₀ plants were fertile and produced T₁ seeds. The T₂ generation was obtained from T₁ seeds. From T₂ plants the DNA was extracted and tested to verify the presence of Gus gene by qualitative PCR and Southern Blot analysis.
ENVIRONMENTS SUSTAINABILITY OF TRANSGENIC CROP: THE CASE OF DROUGHT STRESS IN MAIZE

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drought stress, transgenic maize, gene expression, photosynthesis, proteomics

The environmental sustainability of the main cultures, together with their economic evaluation, is becoming an issue in modern agriculture competitiveness. The extreme exploitation of natural resources, the use of water being a typical example, includes the programmatic decisions for the economic development of a country. Water saving will be necessary in agriculture to face with a greater use for human consumption and for industry.

Crops like maize, which gives high yield and medium profit, is strategic because it impacts both human-animal nutrition, and industry (starch, oil). For this very reason maize is referred to as a commodity for FFP (Food, Feed and Processing).

However maize growth cycle, restricted to the soring and summer periods, is characterised by an high water demand, even if the genetic improvement has allowed to obtain a better environmental adaptability, and there are examples of maize cropped with Deficit Irrigation (DI), or even in dry condition.

The introduction of transgenic maize was finalised to achieve a better environmental adaptability (insect resistance, herbicide resistance). However there is not enough knowledges about drought stress resistance of transgenic plants and about their capacity to adapt to a water limiting condition. At this regard the data available within the notification provided by the seed companies and from other sources, are insufficient.

Furthermore there are not enough information regarding the expression of the transgene in drought stress conditions.

This research adds a useful evaluation criteria for the rational use of genetic resources in a agronomic system in which environmental sustainability is the driving force.

The transgenic maize variety MON 810, is used and compared to non transgenic hybrids Tietar and Famoso. In all varieties the following are analysed:
- functionality of photosynthesis and respiration in normal and drought stress conditions;
- protein synthesis, enzymatic activity, osmotic metabolites;
- expression analysis of candidate genes;
- physiological and yield parameters in greenhouse and in confined conditions.

Since transgenic plants are compared with hybrids characterised by different stress tolerance, it will be possible, for each transgene, to quantify the adaptive response on a reference scale given
by the non transgenic plants; therefore the fitness of each transgene is evaluated in normal and
stressing conditions. Each transgene will be appointed of a ranking for its drought tolerance through
a comparative evaluation against normal crops, both for the general plant behaviour and for the
behaviour of the specific transgene. These informations will constitute a further and rational
contribute to the use of biotechnological crop in modern Italian and European agriculture.
INVESTIGATING RECOMBINANT PROTEIN STABILITY IN THE CHLOROPLAST: THE ROLE OF DISULFIDE BONDS


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chloroplast, disulfide bonds, protein stability, protein quality control, zeolin

In the last two decades there have been growing research efforts on the production of foreign proteins in the chloroplast. The accumulation of a recombinant protein is often increased by chloroplast targeting, even if the wild-type protein is naturally a resident of the cytosol or other compartments. Nevertheless, the results can be unsatisfactory also using these strategies: most likely the chemical environments and repertoire of “folding helpers” of the plastids are not universally optimal and therefore are not suited to any protein.

We have recently produced a chimeric protein, zeolin, that contains domains of the maize prolamin gamma-zein fused with the complete sequence of bean phaseolin. Like gamma-zein, zeolin forms large insoluble polymers in the ER, termed protein bodies (PBs), and accumulates to very high levels in vegetative tissues of transgenic plants. Inter-chain disulfide bonds are a determinant for zeolin PB formation and ER retention. Thus, zeolin is a prototype to study plants as bioreactors for producing pharmaceuticals and other industrial proteins. Unfortunately, when we expressed zeolin in chloroplasts of transplastomic plants, it was largely fragmented and accumulated to unsatisfactory levels, indicating more pronounced proteolytic activity towards zeolin in this compartment than in the ER. Two possible explanations for zeolin poor accumulation in the chloroplast are: (i) zeolin may be highly unstable (very low half-life) because in its native conformation it is recognized as a substrate by chloroplast proteases; (ii) inefficient or partial formation of inter-chain disulfide bonds may cause zeolin misfolding and degradation by the chloroplast protein quality control system. Zeolin does not form PBs in chloroplast, nor do zeolin polypeptides seem to be efficiently linked by inter-chain disulfide bonds, which in the ER are formed by Cys residues of the gamma-zein portion. These results indicate abnormal folding of the recombinant protein, favouring the hypothesis of quality control degradation.

The present research aimed at identifying the defects that limit zeolin accumulation in the chloroplast. By pulse-chase experiments we determined that the half-life of intact zeolin in chloroplasts is around 8 h, indicating that its fragmentation is a slow post-translational process. We are producing transplastomic plants that express a mutated form of zeolin in which the cysteine residues have been replaced with serine residues (zeolinCys-). We previously determined that, when the transgene is inserted into the nucleus, zeolinCys- enters in the ER and is then secreted, indicating that it is unable to form PB but it is not recognized as a structurally defective polypeptide by the ER protein quality control. We want to verify if the absence of disulfide bonds stabilizes zeolin in the chloroplasts or makes it more unstable. Analysis of the transplastomic plants expressing zeolinCys- is in progress. The results will give us information both on the relevance of disulfide bond formation in the chloroplast for zeolin folding and stability and the possible differences between the protein quality control of the two compartments (chloroplast vs ER).
EFFECT OF \textit{AsOXAI} GENE OVER-EXPRESSION ON TRITERPENE SAPONIN BIOSYNTHESIS IN TRANSGENIC BARREL MEDIC


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Medicago truncatula, secondary metabolism, transgenic, triterpene saponin

Triterpene saponins are a group of bioactive compounds abundant in the genus \textit{Medicago} and studied for their biological and pharmacological properties. In the present paper, we evaluated the effects on the production of triterpene saponins in barrel medic (\textit{Medicago truncatula} Gaertn.) by ectopic expression of a novel \textit{Aster sedifolius} (\textit{AsOXAI}) cDNA, a gene encoding for a beta-amyrin synthase, a key enzyme involved in the triterpene biosynthesis. The presence and the expression of the \textit{AsOXAI} gene in different transgenic lines was demonstrated by Southern blot and RT-PCR analyses, respectively. Transgenic \textit{AsOXAI} plants cultured in growth chamber conditions accumulated in the leaves and roots higher amounts of triterpene saponins than control plants. One out of the four \textit{AsOXAI} transgenic lines showed in the leaves a total content of triterpene saponins significantly improved. In particular, transgenic leaves accumulated significant higher values for bayogenin, medicagenic acid and zanistic acid. The level of the two last compounds, which represent the core of the \textit{M. truncatula} leaf saponins, was respectively 1.7 and 2.1 times higher than that observed for the control line. In addition, the production of bayogenin, hederagenin, soyasapogenol E and 2-\beta OH-oleanolic acid in the \textit{AsOXAI} transgenic roots was significantly improved. Under greenhouse conditions, biomass parameters of transgenic \textit{AsOXAI} plants were similar to those observed in the control plants. Interestingly, transgenic roots overexpressing \textit{AsOXAI} showed a better nodulation when compared to the control line. Segregation analysis is currently under way in order to evaluate inheritance of the transgene in the T\textsubscript{1} generation.
Viroids are small non-protein-coding circular RNAs infecting plants. They directly interact with their host transcription and RNA trafficking machineries for replication, subcellular localization and spreading and, as a side effect, they frequently elicit diseases. Viroids are useful tools for dissecting structural-functional relationships of RNA in plants. While members of the family Pospiviroidae replicate and accumulate in the nucleus, *Peach latent mosaic viroid* (PLMVd) and the other members of the family *Avsunviroidae* replicate and accumulate in the chloroplast. Interestingly, chloroplast-replicating viroids are some of the few RNAs able to cross the plastid double membrane, suggesting that RNA trafficking pathways remain to be discovered in plants. Further progress into the underlying molecular mechanisms is hampered by the host range of chloroplast-replicating viroids, which is restricted to the very few plants wherein they have been isolated. Because none of these plants is a model organism, we are exploring alternative systems and, more specifically, the possibility of using for this purpose the non-host *Nicotiana tabacum*. As a first step in this direction, we have generated transplastomic plants with head-to-tail dimeric constructs of PLMVd under the control of a promoter that can drive transcription by the two known chloroplastic RNA polymerases: the plastid-encoded polymerase (PEP) and the nuclear-encoded polymerase (NEP). Analysis by Southern-blot hybridization has identified several homoplasmic lines, and preliminary results from Northern-blot hybridization and RT-PCR indicate that the corresponding transcripts are expressed in some of them. Additional work should clarify whether these primary transcripts are correctly processed into the viroid circular forms, and whether they then act as templates for RNA-RNA transcription like in the natural host. This information may be relevant for understanding the limited host range, which could result from specific access into the chloroplast or from specific factors needed for replication. Transplastomic systems may therefore serve not only for expressing foreign proteins but also for getting insights into the behaviour of non-protein-coding RNAs.
ENZYME REPLACEMENT THERAPY: PRODUCTION OF \( \alpha \)-MANNOSIDASE IN TRANSGENIC TOBACCO PLANTS

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\( \alpha \)-mannosidosis, Nicotiana tabacum, \( \alpha \)-mannosidase, enzyme replacement therapy

\( \alpha \)-Mannosidosis is a rare lysosomal storage disease with autosomal recessive inheritance that leads to mental and physical deterioration. This pathology is due to progressive accumulation of undegraded oligosaccharides inside lysosomes. The deficiency of the \( \alpha \)-mannosidase (LAMAN) enzyme, which normally cleaves \( \alpha \)-linked mannose residues from glycoproteins during their ordered degradation, is the cause of the disease. The enzyme contains 1011 amino acids (108 kDa), including the 49 N-terminal residues which constitute the signal peptide. It is synthesised as a single chain precursor and sorted to the lysosomes where is processed into three glycopeptides of 70, 42 and 15 kDa. In humans, the 70 kDa peptide is further partially proteolysed into three more peptides that are joined by disulfide bridges.

The objective of this study is to provide a plant-based method for the production of LAMAN to be used in “enzyme replacement therapy” (ERT). We report the expression of the human \( \alpha \)-mannosidase gene in stable transformed tobacco plants. Two different constructs were produced: in the first one, pROK8-LAMAN, the full-length cDNA coding sequence of \( \alpha \)-mannosidase was used. The gene was under the control of the rbcS (rubisco small subunit) promoter and NOS (nopaline synthase) terminator. In the second one, pGreen-LAMAN, the \( \alpha \)-mannosidase cDNA expression was controlled by the CaMV 35S (cauliflower mosaic virus) promoter and terminator. Moreover, the original 49 N-terminal signal peptide was replaced by a specific plant signal peptide from PR1 protein and the FLAG epitope was added at the C-terminus of the protein.

Even if the \( \alpha \)-mannosidase gene harboured by the transgenic pROK8-LAMAN tobacco plants showed a good transcription efficiency, no detectable levels of the corresponding enzyme were obtained both in Western blot and enzymatic assays.

On the contrary, pGreen-LAMAN tobacco transformed plants expressed the \( \alpha \)-mannosidase enzyme. The main signal detected in Western blot experiments using the anti-FLAG antibody had a molecular mass of about 110 kDa corresponding to the entire protein precursor, indicating that the protein was correctly synthesised. Western blot experiments using antibodies specific for the \( \alpha \)-mannosidase enzyme revealed several signals corresponding to the single chain precursor and glycopeptides derived from precursor proteolysis. Transformed plants expressing the protein exhibited an enzymatic activity significantly higher then the untransformed tobacco plants. The recombinant enzyme showed biochemical features comparable to those of the human enzyme. Our long-term goal is to offer a new therapeutic approach for \( \alpha \)-mannosidosis.
MODIFICATION AND IMPROVEMENT OF A PLASMID VECTOR FOR THE PRODUCTION OF ANTIGENIC MOLECULES IN GM TOBACCO, FOR VETERINARY USE

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Matrix Attachment Region, Rb7, Nicotiana tabacum, Staphylococcus aureus

The production of important molecules (as subunit vaccines) in plants is increasingly considered for relevant advantages: low costs of production, purification and delivery, no risks of contamination by pathogens and high scale production, but improvement and enhancement of transformation techniques are needed. MAR/SARs (Matrix/Scaffold Attachment Regions) are DNA sequences that have been reported to create a network with the proteinaceous fibrils of nuclear matrix, organizing chromatin into a series of topologically isolated loop domains of 5-200 kb. These sequences may influence the conformation of transgenes and their expression, possibly reducing or eliminating some forms of gene silencing.

Our research is addressed at the production of plant derived antigens, to be used in veterinary prophylaxis. In this field, the optimisation of transgene expression is crucial, also because of the necessity of plant containment during the whole cultivation period.

In particular, we sub-cloned Rb7, a MAR sequence from Nicotiana tabacum, in the binary vector pAMPAT, inside t-DNA close to LB and RB terminations, in its two possible orientations. The vector expression cassette carries a 511 bp portion of Fib, encoding Fibrinogen Binding Protein, from Staphylococcus aureus, under the control of 35SS constitutive promoter. The Fib protein fragment was proved to be effective against S. aureus mastitis in dairy cattle. Nicotiana tabacum, var. Samsun was transformed via Agrobacterium tumefaciens with the four constructs carrying Rb7 elements in all their possible combinations. Statistical analysis was performed after four different experiments, showing an enhanced transformation efficiency for MAR-containing constructs (higher shoot number and shorter shooting time). Molecular and immunological analysis on transformed plants are now in progress, to define the transgene copy number and the resulting protein expression level.
In the last years a remarkable increase in allergic and inflammatory diseases was highlighted. In industrialized countries about 20-25% of living people suffer for IgE mediated allergic diseases. Today allergy immunotherapy is usually performed with natural allergen extracts composed of complex mixtures of several proteins, difficult to standardize and causing cross reactivity.

Recombinant allergens allow to determine the exact sensitization profile of certain individual and this is a prerequisite to select those allergens against which a patient is sensitized for setting up the specific immunotherapy.

House dust mites of Dermatophagoides species (e.g. Dermatophagoides pteronyssinus) are associated with various allergic disease. Der p 1 of the D. pteronyssinus is an allergenic protein considered to be one of the major allergens. On the contrary, even if Der p 10 is a minor allergen its high cross-reactivity with allergens found in a variety of sea foods make it very interesting to be studied.

The expression of Der p1 and Der p 10 allergens mediated by potato virus X (PVX) in Nicotiana benthamiana plants is here reported together with a strategy of Flag and 6his-tag sequences fusion to the N and C-terminus region of Der p1 and Der p 10 cDNAs. The use of Agroinfiltration system to infect N. benthamiana plants is also reported. RT-PCR analysis carried out on cDNAs obtained from mRNA extracted from infected plants and Western blotting with monoclonal anti-Flag and anti-6his antibodies is reported and discussed.
OLEOSINS AS A CARRIER OF ANTIGENS IN PLANT-DERIVED VACCINES

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oleosin, molecular farming, plant-derived vaccines

Transgenic plants have potential advantages over conventional expression systems for the production of recombinant antigens for the formulation of subunit vaccines. In particular, seed-targeted expression is attractive as proteins can accumulate stably. However, the purification of recombinant proteins may be difficult due to the complex proteome of this natural storage site.

Oleosins are hydrophobic plant proteins associated with small subcellular organelles extremely abundant in some seeds and termed oil bodies. These organelles can be easily fractionated from other cellular components through a process of flotation-centrifugation. Exploiting oleosins as carriers of foreign proteins represents an efficient mean to enhance accumulation and purification from seeds.

Transgenic *Arabidopsis thaliana* plants expressing sequences derived from Human Immunodeficiency Virus type 1 as fusion with a sunflower oleosin, have been engineered. Plants have been both genetically and biochemically characterized to verify the expression of the fusion protein. Subsequently, oil bodies have been purified from transgenic *Arabidopsis* seeds and their protein constituents have been further analyzed to verify the presence and integrity of the chimeric oleosins.

Work is in progress to verify the immunological properties of antigens produced by this novel approach in an attempt to extend the range of plant-derived vaccines.
TRANSIENT EXPRESSION OF A GRAPEVINE STILBENE SYNTHASE GENE IN TOBACCO PROTOPLASTS

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stilbene synthase, grapevine, transformation, tobacco

Resveratrol (trans-3,5,4′-trihydroxy-stilbene) is a naturally occurring phytoalexin and antioxidant produced by a restricted number of plants such as grapes, peanuts and mulberry.

Stilbenes are phenylpropanoid derivatives and their biosynthesis is induced by fungal infection and abiotic stress such as wounding and UV irradiation. The physiological functions of most stilbenes, especially resveratrol, are well recognized. The health-related proprieties of resveratrol were indeed investigated intensively over the past two decades and numerous biological activities have been attributed to this molecule rendering very interesting its production by metabolic engineering. The last step of resveratrol biosynthesis is catalyzed by the action of stilbene synthase multigenic family (STS). Likewise chalcone synthase (CHS), the key enzyme in flavonoid biosynthesis in plants, stilbene synthase uses the same substrates but produces different products. Therefore, a single STS gene is sufficient to synthesize resveratrol in heterologous plant species.

Tobacco has a long history as a successful crop system for molecular farming and is therefore one of the strongest candidates for the large-scale production of pharmaceutical macromolecules.

The major advantages of tobacco include the well-established technology for gene transfer and expression, high biomass yield, selfing reproduction and existence of a large-scale processing infrastructure.

The aim of our research is the production of stable transgenic tobacco plants to be used as bioreactors for the molecular farming of resveratrol.

To this target an accurate study of stilbene synthase multigenic family was performed. We used transient expression to verify the expression construct and for localization analysis of STS in tobacco leaves before proceeding to produce transgenic plants. For this purpose a GFP-construct with a stilbene synthase gene isolated from a grapevine cultivar showing high resveratrol production was generated by Gateway® technology. The construct was utilized to transform Nicotiana tabacum protoplasts where it was expressed at the cytosolic level.

Future goal will be the agroinfiltration of tobacco leaves and the regeneration of transformed plants. The expression and activity of the stilbene synthase gene will be analyzed by determining mRNA accumulation and the detection of resveratrol and resveratrol derivates in the transgenic lines will be carry out by HPLC analysis.
CHARACTERIZATION OF ANTIFUNGAL RECOMBINANT ANTIBODIES EXPRESSED IN PLANTS


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therapeutic antibodies, fungal disease, plant farming

Fungal diseases caused by opportunistic fungal agents are dramatically increased in the recent years, in particular candidiasis and aspergillosis carry high morbidity and mortality in immunocompromised hosts. In a recent work [1] a specific anti-ß-glucan monoclonal IgG antibody, named 2G8, was generated in mice. This mAb bound the cell surface of several ß-glucan possessing pathogenic fungi, was able to directly inhibit fungal growth in vitro and exerted a protective effect in vivo against experimental systemic aspergillosis and cryptococcosis as well as against disseminated and vaginal infections by Candida albicans.

Gene engineering has been performed on sequences encoding variable regions of this mAb to devise novel anti-ß-glucan antibodies in different formats: a simple scFv antibody, a human chimeric antibody and a scFv-Fc miniantibody.

In order to exploit the potential of plants as ideal cost-effective expression system, the two full-length antibodies (human chimeric and scFv-Fc) have been transiently expressed in Nicotiana benthamiana plants by the vacuum-agroinfiltration technique. Western analysis of agroinfiltrated plant extracts revealed high expression levels of both chimeric light and heavy chains and of scFv-Fc. The full antibody and the scFv-Fc were purified from leaves by protein A affinity chromatography with very high yields (40 and 50 mg/kg of plant tissue, respectively). The correct assembly of both purified antibodies was evaluated by gel filtration and non reducing SDS-PAGE analysis. In addition, the scFv format expressed only in E. coli periplasm and purified by IMAC, retained the binding specificity of 2G8 mAb in both immunofluorescence and ELISA assays.

All engineered antibodies bound C. albicans and A. fumigatus hyphae. Similarly to what previously found for the protective murine mAb, the human chimeric mAb and the scFv-Fc preferentially recognized ß-glucan molecules in ß-(1,3) configuration and were able to inhibit growth of C. albicans in vitro, in the absence of immune effectors while preventing fungal adhesion to human epithelial cells.

In conclusion, we have generated a panel of recombinant antibodies, derived from a murine protective anti-ß-glucan monoclonal antibody. All recombinant antibody formats retain binding specificity and protection-relevant biological properties of the original mAb and, therefore, could represent potentially valuable tools for both diagnostics and immunoprophylaxis/therapy of human fungal diseases. The high yield obtained in plants confirm the notion that plants represent ultimate bioreactors for the expression of fully functional monoclonal antibodies.
References

POTATO VIRUS X-BASED PLANT-DERIVED VACCINE AGAINST AFLATOXIN B1


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aflatoxin B1, vaccine, PVX, mimotope, Nicotiana benthamiana

Aflatoxin B1, a mycotoxin produced by Aspergillus flavus and Aspergillus parasiticus, is a common contaminant and can occur in a wide range of important raw food commodities causing carcinogenesis and immunosuppression. Moreover, aflatoxins M1 is the hydroxylated form of aflatoxin B1 and this metabolite is produced when cows or other ruminants ingest feed contaminated with this mycotoxin. Aflatoxins M1 is then excreted in the milk and may subsequently contaminate other dairy products. Although the use of pesticides and good agronomic practices can reduce mycotoxin accumulation, its complete elimination is still a major challenge.

An effective strategy to limit the risks deriving from aflatoxin contamination in meat, milk and other derived products could be represented by preventive vaccination. This practice encounters several limitations such as the impossibility to directly administer the active mycotoxin both for its toxicity and the small size (~ 300 Da) which limits its immunogenic potential.

Our work is focused on the identification of a safe non-toxic vaccine against aflatoxin B1 to be tested on animal systems. To this aim, three previously identified peptides (Thirumala-Devi et al., 2001), that mimic the aflatoxin B1 molecule, were produced in plants using the transient expression system based on the potato virus X (PVX) viral vector. The epitope-display strategy using plant virus CP as carriers has been successfully tested for experimental vaccines (Marusic et al., 2001). In addition, the exploitation of plants for the production of therapeutic proteins offers several advantages such as absence of mammalian pathogens, cost effectiveness, large-scale production and relative ease in expression and purification.

All peptides were selected on the basis of specific aminoacid sequence features required for plant virus display (Lico et al., 2006). Hence, mimotopes were expressed on the virus surface as N-terminal fusion to the coat protein (CP) of a mutant PVX, expressing a truncated form of the CP.

The engineered viral vectors were used to inoculate Nicotiana benthamiana leaves and the capacity of the chimeric viruses to move systemically and infect the whole plant has been evaluated. Only one out of the three constructs tested, named 24-6_1, caused systemic infection and was further characterized by RT-PCR and Western.

The recombinant PVX carrying the 24-6_1 mimotope, purified from leaf tissue will be used to immunize mice and to evaluate its capacity to induce an immuno-response specific for the aflatoxin B1. The success of a preventive vaccination against aflatoxin B1 should also permit the reduction of aflatoxin M1 in milk, meat and derived products.
References


CHIMERIC POTATO VIRUS X PARTICLES ACTIVATE INFLUENZA VIRUS-SPECIFIC CD8+ T CELLS IN MICE

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plant virus, peptide vaccine, influenza virus, cytotoxic T cells

Plant virus based expression strategies are the object of intensive investigations as they represent cost-effective, highly scalable and safe systems for the production of recombinant proteins for biopharmaceutical purposes. Plant viruses-based approaches have also been used to produce peptide (epitope)-based vaccines, by the construction of plant Chimeric Virus Particles (CVP) displaying the epitope of interest on their surface as fusion to coat protein (CP) units.

The efficacy of purified, plant-produced CVP in inducing antibody responses specific to the displayed peptide has been extensively demonstrated. We are presently evaluating the capability of the plant virus Potato Virus X (PVX) to elicit specific CD8+-mediated T cell responses. To this aim we have produced in Nicotiana benthamiana plants and purified PVX CVP displaying a D^b-restricted epitope derived from the nucleoprotein (NP) of influenza A virus. Several features have been considered to obtain correctly assembled CVP, able to move systemically and to be correctly processed by antigen presenting cells (APC).

The immune response induced by the plant-produced CVP has been evaluated in mice. The results of IFN-γ ELISPOT assays demonstrate that PVX CVP activate specific CD8+ T cells. Noteworthy, the best response is obtained without adjuvant co-delivery. Serum antibody titration indicates that this could be due to the reduced response against the viral carrier obtained in these conditions.

Our data demonstrate that plant CVP can reach MHC class I pathway of presentation despite being exogenous antigens, paving the way to the possible use of these plant-derived products as carriers able to target and activate different compartments of the immune system.
HIGH EXPRESSION OF THE VACCINIA VIRUS A27L PROTEIN IN TRANSGENIC AND TRANSPLASTOMIC PLANTS

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smallpox vaccine, transgenic plant, Nicotiana tabacum, chloroplast transformation

Orthopoxviruses (OPV) have recently received increased attention, due to the fear of bioterrorism and to the occurrence of zoonotic OPV outbreaks, highlighting the needs for the development of safer vaccines against smallpox and related viruses. To avoid the use of a live virus, the production of subunit protein-based vaccines is an attractive approach. Transgenic plants are ideal means to produce subunit vaccines being them safe, and less expensive to produce and distribute. We are therefore investigating the possibility to produce the immunogenic A27L protein of vaccinia virus (VACV) in tobacco (cv. Petit Havana). Transgenic and transplastomic tobacco plants were produced, respectively, by Agrobacterium-mediated transformation of the nuclear genome and by biolistic transformation of the plastome. Western blot analysis showed the presence of one band of the expected A27L protein size (15 kDa) in the transplastomic and transgenic lines analysed. Two additional bands at ~30 and 40 kDa were displayed only in the transplastomic samples; these bands were thought to be dimers and trimers, a characteristic property of the VACV protein. Indeed, such results were confirmed by electrophoresis of proteins collected on a gradient, which mostly showed the accumulation of the multimeric forms, important for the biological activity of the protein. ELISA analysis demonstrated that the integration of the A27L gene into the chloroplast genome resulted in the accumulation of ~1.6 g/kg fresh weight (equivalent to 18% of TSP). This amount of expression is at least 500-fold higher than the nuclear lines. In fact, values for the transgenic plants analyzed ranged from 1.4 to 3.6 mg/kg fresh weight (0.01-0.04 % TSP). The level of protein accumulation did not decline during leaf development in mature transplastomic plants, suggesting that the protein is stable in transgenic tobacco chloroplasts. Preliminary results indicated that the chloroplast-made A27L protein is recognized by antiserum produced against zoonotic orthopoxviruses. The results obtained herein demonstrate that plastid transformation is a useful for the expression of OPV subunit vaccines. Chloroplast transformation has the additional advantages to co-express multiple antigens in operons and to impose transgene containment through maternal inheritance.