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Oral Communication Abstract – 5C.01

PRODUCTION OF RENEWABLE POLYMERS FROM CROP PLANTS

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Polymers, plastics, rubber, polyhydroxyalkanoate, cyanophicin

Plants produce a range of biopolymers for purposes such as structural integrity, carbon storage, and defense against pathogens and desiccation. Several of these natural polymers are used as food, materials, and increasingly as an energy carrier. In this presentation, we will focus on plant biopolymers used as materials in bulk applications, such as plastics and elastomers, in the context of depleting resources and climate change, and cover technical and scientific bottlenecks in the production of novel or improved materials in transgenic or alternative crops plants. The biopolymers discussed are natural rubber, and several polymers that are not naturally produced in plants, such as polyhydroxyalkanoates, fibrous proteins, and poly-amino acids. In addition, monomers or precursors for the chemical synthesis of biopolymers, such as 4-hydroxybenzoate, itaconic acid, fructose, and sorbitol, are briefly discussed.

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Oral Communication Abstract – 5C.02

GREEN MICROALGAE AND PLANTS EXPRESSING FUNGAL LACCASES USEFUL FOR ENVIROMENTAL AND INDUSTRIAL APPLICATIONS

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Laccase, tobacco, green microalgae, bioremediation

Industrial activities release a wide range of toxic chemicals into the biosphere. Among such chemicals, phenolic compounds represent a major risk for human health and biodiversity mainly due to their relative persistence in the environment. In the Mediterranean area the olive oil industry accounts for about 95% of the entirely world olive oil production. Olive oil mill wastewaters are characterised by low pH and a high content of mono- and polyaromatic compounds that exert microbial and phytotoxic activity. White-rot fungi have been proved to secrete a large array of ligninolytic enzymes which are highly efficient in degrading aromatic compounds, such as lignin peroxidases, manganese peroxidase and laccases. The latter enzymes, have been proved to be useful for industrial applications, such as biobleaching of pulp, organic synthesis and fruit juice process. Our final goal is to enhance the laccase production in green organisms and to evaluate their ability to secrete laccases into the environment.

A biotechnological approach to enhance the efficiency of xenobiotic removal from green organisms is to overexpress genes involved in metabolism, uptake or transport of specific organic pollutants. However, a useful approach is to secrete laccases in the soil or water. The laccase cDNA of the *pox*A1b gene from *Pleurotus ostreatus*, carrying a signal peptide sequence for enzyme secretion and driven by the CaMV 35S promoter, was cloned into a plant expression vector. Nuclear genetic transformation was carried out by co-cultivation of *Agrobacterium tumefaciens* with tobacco cv Samsun NN leaves and cells of five different microalgae accessions belonging to the genera *Chlamydomonas, Chlorella* and *Ankistrodesmus*. Transgenic plants and microalgae were able to express and secrete the recombinant laccase in the root exudates and the culture medium, respectively. In comparison to untransformed controls, the ability to reduce phenol content in OMW solution was enhanced up to 2.8-fold in transgenic tobacco lines and by up to about 40% in two microalgae accessions. The present work provides new evidence for metabolic improvement of green organisms through the transgenic approach useful for environmental and industrial applications.

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Oral Communication Abstract – 5C.03

IMPROVING EXPLOITATION AND SACCHARIFICATION OF BIOMASS FOR BIOCONVERSION

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Crop residue, cell wall degrading enzymes, biofuel, saccharification, agricultural residue

Cell wall recalcitrance to enzymatic hydrolysis is the main bottleneck for the industrial scale-up of biomass processing and bioconversion. Our research may help in overcoming the difficulties of converting plant biomass into usable products. Engineering or selecting plants with altered expression of cell wall related proteins may help either breakdown the components of the cell wall or prevent the cell wall polysaccharides from forming cross-links. Pectin contributes to cell wall rigidity through homogalacturonan (HGA) calcium-mediated cross-links. We have demonstrated that saccharification efficiency of dicot and monocot biomass is improved by reducing the amount of acidic HGA domains through the constitutive expression of a fungal polygalacturonase (PG) or the overexpression of a pectin methylesterase inhibitor (PMEI). We show now that an improved saccharification efficiency without affecting biomass production (as observed previously for the constitutive expression of PG) can be obtained through the conditional expression of genes encoding pectin degrading enzymes, for example a bacterial pectate lyase (*pel1*) or fungal PG genes, at selected stages of development using a chemically- or senescence- inducible promoter. We show also that enzymatic saccharification is improved in different Arabidopsis mutants with a lower content of de-methylated stretches of HGA as compared to the wild type. We propose the use of an immunoassay for detecting unesterified HGA levels as a tool to isolate natural variants with improved saccharification efficiency. We are also performing the analysis of the cell wall components of different varieties of tomato, barley, grapevine and wheat, in relation to their different degrees of saccharification. This will help us to identify useful markers for breeding new varieties suited to food and bioenergy purposes.

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Oral Communication Abstract – 5C.04

USE OF FOURIER TRANSFORM INFRARED SPECTROSCOPY FOR THE CHARACTERIZATION OF ALGAL COMPOSITION AND THE SELECTION OF STRAINS FOR PRODUCTIVE PROCESSES

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Biofuel, lipid, carbohydrates, FTIR spectroscopy, algae

The characterization of biomass is typically a rather lengthy, complex, costly and time consuming endeavor. Because of this, screening of species and growth condition for commercial applications is often limited to few organisms and growth treatments or based solely on literature information. Conversely, Fourier Transform Infrared (FTIR) spectroscopy allows a reliable, fast, and inexpensive assessment of the quality of biomass, without the need for extractive procedures and distinct analytical methods for the different pools. Furthermore, the use of FTIR spectroscopy minimizes sample size, because few cells are needed for each measurement.

Our work was performed on 11 microalgal species belonging to a wide range of taxa. Each of these species was subjected to three growth regimes that differed for the availability of nitrogen (provided as nitrate). We chose this cultural system because an imbalance in the external C:N ratio may appreciably affect C allocation thus allowing the generation of biomass with a variety of stoichiometries.

We developed a new computational method for the semi-quantification of macromolecular pools; this made it possible to partially overcome the difficulties associated with a quantitative use of whole cell FTIR spectroscopy. No obvious relationship was observed between the taxonomy and the C allocation patterns of the 11 algal species. Most species responded to a lower N availability by accumulating lipids or carbohydrates. *Dunaliella parva* and *Thalassiosira pseudonana*, however, were homeostatic with respect to their organic cell composition. The cell lipid content showed a a hyperbolic relationship with cell volume. The FTIR data were selectively validated by comparison with commonly used methods.

Our results confirmed that FTIR spectroscopy is a powerful tool for the assessment of biomass quality and for the rapid screening of large number of species and growth conditions. Since this methodology allows the simultaneous determination of all main macromolecular pools, it can be applied regardless of what end product is targeted.

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Oral Communication Abstract – 5C.05

UTILITY OF BETA-CYCLODEXTRINS FOR ENHANCING ARTEMISININ PRODUCTION IN *ARTEMISIA ANNUA* CELLS

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Artemisia, cell cultures, artemisinin, cyclodextrins, gene expression

Artemisinin is an effective antimalarial compound produced, though at low levels (0.1–1% dry weight), by the aerial parts of the herbal plant *Artemisia annua* L. Due to its antimalarial properties and short supply, efforts are being made to improve our understanding of artemisinin biosynthesis and its production. *A. annua* cell and tissue cultures were also explored for the production of artemisinin, although the yields obtained have been so far quite low. We have recently established *A. annua* cell cultures able to biosynthesize artemisinin and to respond to the elicitation by methyl jasmonate (MeJA). Interestingly, amounts of artemisinin produced by these cultures were also found in the spent culture medium. Cyclodextrins (CDs) are known to increase the water solubility of various compounds, by forming host-guest inclusion complexes. The aim of this work was to evaluate the ability of 2,6 dimethyl-β-cyclodextrins (DIMEB) to enhance artemisinin production in *A. annua* suspension cell cultures. HPLC analysis of artemisinin and Real Time PCR gene expression analysis were carried out in DIMEB treated and untreated cultures. DIMEB induced a 300-fold increase of artemisinin levels in the spent culture medium after a three-day-treatment thus showing to significantly enhance artemisinin production in *A. annua* cell cultures. The addition of MeJA induced a further increase of the artemisinin amounts. The gene expression results obtained did not make it possible to assess a clear correlation between transcript accumulation of the artemisinin biosynthetic genes and artemisinin levels. Further investigations are needed to clarify whether the increase of artemisinin production induced by DIMEB was the result of the enhancement of the artemisinin biosynthetic flux .

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Poster Communication Abstract – 5C.06

PLANT CELL CULTURES FROM *JATROPHA CURCAS*: A POSSIBLE SOURCE OF RENEWABLE ENERGY

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Jatropha curcas, plant cell cultures, Agrobacterium tumefaciens, transformation, DGAT

Negative environmental consequences of fossil fuels and concerns about petroleum supplies have spurred the search for renewable transportation biofuels. Biodiesel is the most used alternative biofuel nowadays, along with bioethanol, in the world. The increasing demand of plant species producing oil rich seeds is leading to the reduction of agricultural areas assigned to crops grown for human nutrition and it is causing food price raise. *Jatropha curcas,* a plant native of Mexico and Central America, has attracted the interest due to its easy adaptability to semi-arid marginal sites, its seeds producing non-edible oil used as a diesel fuel substitute and its role in erosion control. Considering its enormous potential, a large amount of quality planting material is required in the future. The aim of the proposed project is to design reliable protocols to obtain biomass production and to induce lipid accumulation in cell cultures obtained from *J. curcas* explants. The experimental plan led to the establishment of protocols able to stimulate cells proliferation from leaf and seed explants and to the set up of easy and fast clonal propagation by cuttings, organogenesis and somatic embryogenesis. Cell proliferation was obtained in many different conditions of salt and phytohormone combinations in the culture medium. *In vivo* propagation through cuttings was carried out from plants of different age and origin. First results indicate that the hardwood part of the plants is the most efficient in rooting and outliving. Organogenesis via a callus-mediated step and plant regeneration were achieved *in vitro*, but improvements are needed both in propagation techniques and adaptation to natural environment modalities. Some protocols to induce repetitive somatic embryogenesis in *J. curcas* have been applied starting from different explants (leaf, hypocotyl, seed).

Genetic engineering through transformation is a valuable method for the development of oil enriched varieties. Susceptibility of *J. curcas* leaf explants to *Agrobacterium*-mediated transformation was tested, and transformed calli constitutively expressing GFP protein are now available in our laboratory. On this basis, *Agrobacterium*-mediated transformation was planned to insert a gene involved in the plant lipid biosynthesis pathway. The chosen gene encodes for an enzyme, diacylglycerol acyltransferase (DGAT), that catalyzes the final rate-limiting step of the triglycerides condensation. A cloning cassette with *A. thaliana* DGAT gene fused with the eYFP gene, under the control of a constitutive CaMV 35S promoter, has been cloned into a pGreen-based delivery plasmid. *Agrobacterium*-mediated transient transformations are being performed to test the correct expression of the fusion protein.

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Poster Communication Abstract – 5C.07

PRELIMINARY STUDIES OF AN ITALIAN GIANT REED (*ARUNDO DONAX* L.) CLONES COLLECTION

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Biomass, Arundo donax, energy crop

Giant Reed is a perennial grass native of Asia, nowadays spread out in the Mediterranean area, America, Australia and North Africa. It is especially interesting as energy crop because studies conducted in Italy reported a biomass production of about 40 tons of dry matter per hectare. Giant Reed has long been associated with humans and used to satisfy local necessities, such as plant-tutors, walking-sticks, baskets and mats. Its sterility is a serious obstacle for breeding programmes with the aim of increasing productivity and biomass quality for energy conversion. In fact *A. donax* propagation is based on asexual reproduction exclusively through rhizomes and stems nodes; one shoot can sprout from each node stem in water. Although some papers reported a very low genetic variability others described significant differences in phenotypic traits. In this work we collected giant reed clones from all Italian territory: we explored along roads, railway tracks, riversides and riverbeds, scattered uncultivated areas and urban contexts, collecting 90 *Arundo donax* clones (we sampled one rhizome of about 500g with at least one vegetative bud). The rhizomes were planted (2 m between rows and 2 m within row, 20-30 cm deep) at the experimental field of the University of Milan (Landriano area 45°18’N latitude; 9°15’E longitude). We will present a preliminary characterization of this collection under several points of view.

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Poster Communication Abstract – 5C.08

CHARACTERIZATION OF CALABRIA AND SARDINIA MYRTLE USING GENETIC, CHEMICAL AND BIOLOGICAL MARKERS

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Myrtus communis, genetic diversity, chemotipes, biological activity, AFLP

Myrtus communis is a shrub that belongs to the Myrtaceae family, it is widespread in the Mediterranean basin primarily near the coastal regions and on the islands. It is known for its essential oil extracted from leaves and mature fruits, rich in linear, cyclic, and bicyclic monoterpenes. In Calabria and Sardinia *M. communis* historically represents an important natural source, and possesses an interesting potential, mainly in food and flavor industries. However, the intra-specific variability could dramatically affect the chemical composition of a plant species, therefore great attention has been recently devoted by manufacturers toward to the characterization of wild-type plants, with the aim of detecting ecotypes commercially exploitable.

In this work ecotypes of myrtle were collected in several localities of Calabria and Sardinia, and analysed for *i*) genetic relationships by means of AFLP markers; *ii*) chemical diversity, assessing the content in myrtenol, linalool, and eucalyptol, the compounds that better represent the apolar portions of the leaves extracts; *iii*) the biological activity activities (antioxidant, antibacterial, and antifungal) of the extracts.

The genetic analysis evidenced that Calabria and Sardinia ecotypes are genetically distinct.

Calabrian samples evidenced an average total monoterpene content almost 47% lower than that detectable in Sardinian samples. The qualitative and quantitative determination of the single compounds in all the myrtle samples led to conclude that three chemotypes are present: eucalyptol, linalool and myrtenol. Eucalyptol chemotype revealed to be slightly prevalent in Sardinia, while in Calabria it showed a presence equal to linalool chemotype. In both the regions, myrtenol chemotypes were less abundant. In some cases a relationship between the antioxidant and antibacterial activity and the myrtenol, linalool, and eucalyptol quali–quantitative assessment, was established. Antifungal activity was always very weak and far to prospect possible myrtle applications taking advantage of this biological capacity. However, it is evident that a more consisting correlation could be done only with a deeper phytochemical investigation, targeting other secondary metabolites (i.e. polyphenols).

Anyway, this work represents the first researching step on Sardinian and Calabrian myrtle biodiversity and suggests continuity in the investigation, in particular for those samples which

already evidenced a possible strong relation between biological activity and phytochemical characterization.

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Poster Communication Abstract – 5C.09

IDENTIFICATION OF *ARTEMISIA UMBELLIFORMIS* GENOTYPES SUITABLE FOR CULTIVATION

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Artemisia umbelliformis, AFLP markers, genotype fingerprinting, genepì liqueur

Artemisia umbelliformis Lam. is an herbaceous plant belonging to the *Asteraceae* family and growing wild at an altitude of between 2,000 and 3,700 m (a.s.l.). Its flowers are mainly used for the production of “Genepì”, an highly prized liquor of bitter taste whose peculiar flavour is given by the plant volatile constituents and sesquiterpene lactones. The indiscriminate picking of the flowers undermines the survival of the species in nature and its harvesting is banned in Switzerland and Italy and strictly regulated in France.

A. umbelliformis is at present grown in North-western Alps, but flower production does not meet market needs; thus it is important to select high productive genotypes representative of the genetic variation at present in cultivation.

Within the Interreg ALCOTRA ‘*Alpi Latine Cooperazione Transfrontaliera* Project “GENEALP – Genepì delle Alpi e altre piante officinali” we performed an AFLP-based assessment of the genetic architecture in five *A. umbelliformis* ecotypes grown in as many locations (Val Gesso, Marmora, Elva, Gran Paradiso and Val Chisone) as well as a selected (RAC12) and a natural population (Wild). The evaluation of the genetic variability between and within ecotypes/populations enabled to detect the distribution of the analyzed genotypes into three main clusters. Ecotypes Val Chisone, Elva, Gran Paradiso and Valle Gesso were chosen as representative of the genetic variability present in cultivation and referred as "Occitan" ecotypes. Within each of these ecotypes, 10 plants were selected, according to the production observed at the time of flowering (number of flowers per plant).

PCO analysis based on their AFLP-fingerpring confirmed the genetic differentiation among "Occitan" ecotypes and made it possible to identify 10 genotypes (plants) which will be used as mother plants to obtain *in vitro* clonal populations. This clonally propagated material will subsequently be evaluated for cultivation.

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Poster Communication Abstract – 5C.10

A CANDIDATE GENE APPROACH FOR IDENTIFYING QUANTITATIVE TRAIT LOCI AFFECTING TOMATO BIOMASS CHEMICAL COMPOSITION

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Tomato, biofuel, sugar metabolism, introgression lines

Tomato is the most important vegetable cultivated in Italy and a well-studied crop species in terms of genetics, genomics, and breeding. In particular, a permanent mapping population composed of 76 introgression lines (ILs) of *L. pennelli* have been used extensively for identification and mapping of many biologically and agriculturally important traits. Considering its wide cultivation in our country the possibility to use their biomass for biofuel production is being explored. Successful use of biomass for biofuel production depends on not only pretreatment methods and efficient processing conditions but also physical and chemical properties of the biomass. In this study, 4 genes previously located on the tomato map using an introgression lines, involved in sugar metabolism were selected and further analyzed. Expression changes among selected ILs (IL 4-4; IL 6-2; IL 7-2) were assessed by Real-time PC. The ADH2 gene shows expression significantly increased in fruit than in leaf while the Ppc1 gene is highly expressed in leaf IL 6-2. Comparison between phenotypic characteristics and candidate gene loci indicated a few candidate genes may influence the variation of biomass composition.

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Poster Communication Abstract – 5C.11

RHIZOSECRETION OF CELLULASES FOR BIOETHANOL PRODUCTION

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Bioethanol, cellulases, rhizosecretion, hydroponic colture, Nicotiana tabacum

The most common renewable fuel today is ethanol derived from corn grain (starch) and sugar cane (sucrose). It is expected that there will be limits to the supply of these raw materials in the near future, therefore lignocellulosic biomass is seen as an attractive feedstock for future supplies of ethanol. However cellulose is highly crystalline and compact making it very resistant to biological attack and much more difficult than starch to enzymatically degrade to fermentable sugars. Moreover the presence of non-glucose sugars in the feedstock complicates the fermentation process because conversion of pentose sugars into ethanol is less efficient than conversion of the hexose sugars. Consequently the cost of producing ethanol from biomass is higher than production from starch. In order to optimise the process the enzymes used for biomass hydrolysis must become more efficient and far less expensive.

Plants have been shown to be suitable for production of many recombinant proteins. Indeed, numerous recombinant proteins have been produced in various plant tissues and targeted to different subcellular compartments, such as cytoplasm, endoplasmic reticulum (ER), or apoplastic space. However, the extraction and purification of proteins from biochemically complex plant tissues is a laborious and expensive process and a major obstacle to large-scale protein manufacturing in plants. Root secretion can be successfully exploited for the continuous production of recombinant proteins in hydroponic coltures, in a process named "rhizosecretion."

Here we show the results obtained using tobacco plants transformed with genes encoding bacterial or fungal cellulases secreting enzymes in the apoplast and in the rhizosphere.

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Poster Communication Abstract – 5C.12

CYNARA CARDUNCULUS L.: FROM VEGETABLE TO ENERGY CROP

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Cultivated cardoon, biomass, energy crop, QTLs

Cynara cardunculus (*Asteraceae*) is native to the Mediterranean basin and includes: the globe artichoke (var. *scolymus*) and the cultivated cardoon (var. *altilis*), grown respectively for the production of immature inflorescences and fleshy stalks consumed as vegetables, as well as their progenitor wild cardoon (var. *sylvestris*).

The species is perennial and has great potential as a source of renewable energy, thanks to its productivity of lignocellulosic biomass. The calorific value of the three *C. cardunculus* taxa is analogous, however cultivated cardoon has the highest biomass yield, which can reach up to ~19t/ha (energy value ~ 17 MJ/kg).

Within the Project 'CYNERGIA' (funded by the MIPAAF), 9 commercial varieties and local ecotypes of cultivated cardoon are being evaluated for biomass yield (10,000 pt/ha) in three environments located in North and South Italy. During the first year the biomass production reached values up to 8.3 t/ha (dry matter), which is expected to high increase during the second and following years. The estimated Net Energy Balance (NEB) reached values in excess of 100 GJ/ha, with an energy input of 29 GJ/ha. The first year estimated energetic efficiency (NER) was 3.9, it will increase starting from the second year due to both higher biomass production and not need for crop establishment (~ 50% of the energy costs).

A double pseudo-testcross mapping strategy has been previously applied to developed molecular linkage maps based on the F_1 progeny from the cross between a genotype of globe artichoke 'Romanesco C3' (female parent) and one of cultivated cardoon (Altilis 41), contrasting in biomass yield. About one thousand markers were attributed to 17 major linkage groups and 384 SNP markers will be soon mapped by the GoldenGate Assay platform (Illumina). The segregating progeny has been already characterized in autumn 2010 for traits associated to biomass production, and a second round of characterization in year 2011 is in progress. This will make it possible to identify Quantitative Trait Loci related to bio-energy traits, with the goal of future implementation of marker assisted breeding programs.

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Poster Communication Abstract – 5C.13

RECOVERY OF THERMOSTABLE CELLULASES FROM WOODLAND SOIL BY MEANS OF METAGENOMIC APPROACHES

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Cellulose, cellulase, endo-β-1,4-glucanases, Bacillus subtilis, metagenomic

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compound in the biosphere. It has enormous potential as a renewable source of energy and has attracted the interest of biotechnologist who wish to use it as a source of fuels and chemicals. Currently, most biofuel is in the form of ethanol generated from starch or sugar, but this can meet only a limited fraction of global fuel requirements. Conversion of cellulosic biomass, which is both abundant and renewable, is a promising alternative. Unfortunately, cellulose is naturally resistant to biological degradation because of its rigid structure and insolubility. The complete degradation of this polysaccharide into fermentable sugar involves the synergistic action of three different class of enzymes collectively known as cellulases: endo-β-1,4-glucanases, exo-β-1,4-glucanases, and β-glucosidases. Tolerance to high temperature is a desirable property of the enzymes, and the degradation of cellulose at high temperature shows several benefits, among which increased cellulose activity, lessened energy cost for cooling, and decreased risk of contamination. In this study we succeeded in isolating thermostable cellulases from woodland soil samples by means of metagenomic approaches. Based on a known thermostable endo-β-1,4-glucanases from Bacillus subtilis available online (A.N.: FJ464332) we developed primer pairs that were tested on the bacterial DNA directly recovered from the soil samples. Amplified fragments of the expected sizes were recovered from the gel, cloned and the single clones sequenced. Twenty five different cellulase sequences from B.subtilis were isolated. All the obtained sequences had nucleotide and aminoacid variations with respect to the online sequence. Ten out of twenty five sequence were cloned in expression vector pET28b and then successfully extracellularly expressed in Escherichia coli BL21. The activity and the thermostability of the enzymes were tested, two of the isolated genes show high performance at elevated temperature making them good candidates for biotechnological applications involving cellulose modification.

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Poster Communication Abstract – 5C.14

CHARACTERIZATION OF HUMAN ALPHA-MANNOSIDASE SECRETORY PATHWAY IN TOBACCO PLANTS

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Biopharmaceuticals, lysosomal enzyme, mannosidase, mannosidosis, Nicotiana tabacum

Traffic of α-mannosidases to the lysosomal or vacuolar compartment follows alternative routes in different kingdoms; In animal cells, these hydrolases are transported to lysosomes by the mannose 6-phosphate pathway, in plants, vacuolar α-mannosidase is targeted to its final destination via the classic secretory pathway involving the ER-Golgi system, whereas in yeast vacuolar delivery of α-mannosidase can be reached by both cytoplasm to vacuole targeting (Cvt) and autophagy pathways. Recently, tobacco plants expressing a human lysosomal α-mannosidase (MAN2B1) have been obtained. In tobacco leaves, the recombinant enzyme was found to be N-glycosylated and localised in vacuolar compartments, even if the plant counterpart of the mannose 6-phosphate pathway is not known. We then tried to understand what kind of mechanism uses this human protein in the plant to reach the vacuole. To study the traffic of the precursor MAN2B1 polypeptide, transgenic leaf protoplasts were incubated in the presence of the fungal toxin brefeldin A (BFA) and then pulse-chase analysed. Protoplasts were homogenated and immunoprecipitated with anti-MAN2B1 antiserum. BFA negatively affects Golgi-mediated protein traffic and in presence of this toxin the traffic of proteins to the vacuole is inhibited. Conversely, the 110-kD MAN2B1 precursor is substantially not affected by the addition of BFA and this suggests that the traffic to the vacuole of the precursor MAN2B1 polypeptide is not dependent on Golgi-mediated delivery. To confirm the results of the pulse-chase analysis, an aliquot of the BFA-treated protoplasts was subjected to microscopy analyses. Immunolocalization of MAN2B1 indicated that the protein was mainly detectable as small and delimitated structures, which did not change after BFA addition, confirming the existence of a MAN2B1 route to vacuoles which does not pass through the Golgi apparatus. We are performing other analyses on transformed tobacco seeds to characterize the MAN2B1 route to vacuoles. Moreover, the MAN2B1 polypeptide does not contain known plant sorting signals that would direct it to the vacuole. Therefore, we are investigating which structural part of the protein acts as a vacuolar targeting signal.

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Poster Communication Abstract – 5C.15

IN PLANTA PRODUCTION OF *DERMATOPHAGOIDES PTERONYSSINUS* Der p 10 ALLERGEN

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Dermatophagoides pteronyssinus, allergens, Potato virus X, Agroinfiltration

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 determining 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. *D. pteronyssinus*) are associated with various allergic diseases. One of the most important allergens is Der p 10 which shows a high cross-reactivity with allergens found in a variety of seafood.

We have transiently expressed Der p 10. To do so, a modified version of the potato virus X (PVX) was used for agroinfiltrating *Nicotiana benthamiana* plants. In particular, FLAG-6His tag was fused to ether the N or C-terminus region of the Der p 10 cDNA. RT-PCR analysis carried out on cDNAs retrotranscribed from mRNAs extracted from infected plants, Western blotting with monoclonal anti-Flag and anti-6his antibodies, and rDer p 10 immunological characterization is reported and discussed.

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Poster Communication Abstract – 5C.16

EXPRESSION AND PURIFICATION OF A MUTATED FORM OF HUMAN GAD65 FROM TRANSGENIC TOBACCO LEAVES

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T1DM, GAD65mut, Nicotiana tabacum, purification

Type 1 insulin-dependent diabetes (T1D) is caused by the autoimmune destruction of insulin-secreting beta cells, leading to a life-long insulin deficiency (Gepts, 1965). The young age of affected patients, the need for insulin therapy and the high prevalence of late-onset complications make T1D a major health problem.

The 65 kDa isoform of glutamic acid decarboxylase (GAD65), present on pancreatic islet beta cells, is one of the major autoantigens implicated in the development of human T1D (Baekkeskov *et al*., 1982) and it has been recently demonstrated in a Phase II Clinical Trial that two injections of the molecule can give protection to T1D. The final therapeutic aim for T1D is primary prevention because of the difficulty in identifying people at risk of T1D within the population. Vaccination studies and subsequent vaccination treatment of a large number of people would require a huge amount of purified protein, but the current production platforms are too expensive and unable to provide enough GAD65 to meet global demand.

GAD65 has previously been expressed in *Nicotiana tabacum* plants but yields were disappointing (maximum 0.25% of total soluble protein, TSP) (Porceddu *et al*., 2009; Ma S. *et al*., 2004; Wang *et al*., 2008; Avesani *et al*., 2003). In order to improve the recombinant protein expression level, we expressed a mutated form of the molecule with no catalytic activity (hGAD65mut), hypothesising that the enzymatic activity might interfere with its accumulation.

The mutated form of the molecule we used was previously described (Hampe *et al*., 2001) and it was characterised by the substitution of the amino acid residue responsible for cofactor binding in the catalytic site.

We showed that GAD65mut accumulates to higher levels in transgenic plants (2.2% TSP) than GAD65 (Avesani *et al*., 2010), suggesting that the catalytic properties of GAD65 could contribute to its poor yields.

A 1% total soluble proteins (TSP) yield of a recombinant protein in transgenic plant is considered to be the minimum required to make the extraction of a plant-derived pharmaceutical protein economically viable. Given that we obtained GAD65mut maximum yield of about 2.2% TSP, the highest-expressing GAD65mut plants allowed us to begin the set up of all steps and parameters for the purification process of the recombinant protein from plant tissue and the evaluation of plant platform capacity to meet global demand of GAD65 protein for vaccination studies and treatment, in comparison to other expression platforms.

The results obtained during the set up of the extraction and the first steps of the purification protocol are discussed.

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Poster Communication Abstract – 5C.17

EXPRESSION OF N-TRUNCATED GAD65mut FORMS IN A PLANT-BASED PLATFORM

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T1D, GAD65, Nicotiana benthamiana, transient expression

Type 1 diabetes (T1D) is an autoimmune disease characterized by the T-cell mediated destruction of insulin-secreting pancreatic β-cells, causing the need of life-long insulin therapy. The 65 kDa isoform of glutamic acid decarboxylase (GAD65) present in the pancreatic cells is one of the major autoantigen involved in disease development. In the last years antigen-specific immunotherapy (ASI) based on the delivery of GAD65 has emerged as an appealing approach for treating T1D; recent phase II clinical trials have shown that human administration of two injections of 20 µg of alum-formulated GAD65 lead to a significant preservation of residual insulin secretion without serious adverse effects. Large-scale phase III confirmatory studies are underway in Europe and in the USA. The major disadvantage of this approach is the high-cost associated with the current molecule production system based on Baculovirus/insect cells (500,000 €/g). In the perspective of the use of GAD65 for autoimmune treatment a cost-effective recombinant system for the production of the immunoreactive protein would be highly desirable.

It is well documented that GAD65 undergoes some post-translational modifications in the N-terminal domain that result in a firmly membrane-anchored protein, which is highly hydrophobic. In vitro, GAD65 requires detergent to be solubilised. However, because detergents are extremely cytotoxic, a detergent-free preparation is mandatory for vaccination; moreover the presence of detergents can complicate the purification process. The production of a soluble form of the protein would simplify the downstream processing of the molecule and, eventually, the final pharmaceutical formulation.

We have previously shown that GAD65 and a mutated catalytically-inactive form of the protein (GAD65mut) can be expressed in transgenic tobacco plants. GAD65mut accumulates 10-fold higher than GAD65 and retains the immunogenic properties.

In order to develop a system for the high-efficient production and purification of GAD65, we engineered GAD65mut to various extents to obtain soluble forms of the molecule. In the present work we describe and discuss the solubility and accumulation levels of three N-truncated forms of GAD65mut in comparison with full-length GAD65mut and GAD65 in a plant-based platform. This system, based on transient expression in *N. benthamiana*, was chosen for its high-throughput and fast expression of molecules.