

Oral Communication Abstract - 5.01

PLANTS WITH TRANSGENIC PLASTIDS

R. BOCK

Max Planck Institute for Molecular Plant Physiology; Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany - rbock@mpimp-golm.mpg.de

plastid transformation, metabolic engineering, molecular farming, biotechnology, containment

Plant genetic engineering will likely contribute to the required continued increase in agricultural productivity during the coming decades. Moreover, plants can potentially provide inexpensive production platforms for pharmaceuticals and nutraceuticals. With the advent of technologies to alter the genetic information inside plastids (chloroplasts), a new attractive target for genetic engineering has become available to biotechnologists. There are considerable attractions of the plastid genome as a target for the expression of foreign genes. These include (i) the plastids' potential for high-level foreign protein expression, (ii) the possibility of transgene stacking through expression of multiple genes from operons, and (iii) the absence of position effects and epigenetic gene silencing mechanisms (Bock, 2001; Khan and Bock, 2004). From the biosafety perspective, the major attraction is the exclusively or predominantly maternal inheritance of the plastid genome in most crop plants, greatly reducing the risk of uncontrolled pollen spread of transgenes and thus allaying concerns over environmental consequences of GM crop cultivation.

Biotechnological applications of plastid genome engineering have long been hampered by the lack of workable plastid transformation systems for major crops. We have recently developed a plastid transformation system for tomato, a food crop with an edible fruit (Ruf et al., 2001). Transplastomic tomato plants are fertile and express foreign genes to high levels even in non-green plastids (chromoplasts) of the ripe tomato fruit. This system facilitates safe metabolic engineering and efficient production of nutraceuticals and biopharmaceuticals in consumable plant organs. The state of the art in plastid transformation technology, newly developed tools for transgene expression in plastids and selected applications in metabolic engineering, resistance management and molecular farming will be discussed.

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IMPORT OF MACROMOLECULES IN PLANT MITOCHONDRIA

T. SALINAS*, A. PLACIDO**, M. ZAEPFEL*, AM DUCHENE*, L. MARECHAL-DROUARD*

*) Institut de Biologie Moléculaire des Plantes, UPR2357 du CNRS, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France

***) Dipartimento di Biochimica e Biologia Molecolare - Università di Bari, CNR, Via Orabona 4, 70126 Bari, Italy

import, mitochondria, DNA, tRNA, proteins

The plant cell has a particular feature to contain three instead of two compartments where translation events take place: the cytosol, mitochondria and chloroplasts. To be active, each of these translational machineries must possess complete sets of aminoacyl-tRNA synthetases (aaRS) and tRNAs. During evolution, chloroplast and mitochondrial genomes of plants have lost a number of corresponding genes. All aaRS are now encoded in the nucleus and imported into the organelles. Using *in silico*, analyses and *in vivo* and *in vitro* experiments, we have shown that dual targeting is the rule for organellar aaRS. This situation represents an unprecedented degree of cross-compartment sharing of aaRS that has occurred during evolution.

All chloroplastic tRNAs are encoded in plastid DNA. In contrast, the mitochondrial population of tRNA is much more complex. MtDNA-encoded tRNAs characterized by typically mitochondrial features (called "native") are not sufficient to decode all 61 codons of the genetic code and mitochondria uses two other sources to complete the tRNA set. One portion of missing tRNAs is replaced by expressed tRNA genes carried by chloroplast DNA fragment inserted into the mitochondrial genome during evolution. The second one is provided by import of nuclear DNA encoded tRNAs from the cytosol. At least one third of the mitochondrial tRNAs of higher plants are nuclear-encoded and imported into mitochondria. This phenomenon is not plant specific and the import of tRNAs encoded in the nucleus is essential for proper protein translation within mitochondria of a variety of evolutionary distant organisms. However, in spite of a broad occurrence of this pathway among organisms, mechanisms governing RNA targeting into mitochondria remain poorly understood. By developing *in vivo* approaches, biochemical studies and an *in vitro* tRNA import system, we are trying to elucidate the mechanism involved in plants. Moreover, in an attempt to study tRNA stability once internalized into the organelles, we have developed a strategy based on direct DNA uptake into the mitochondria, this strategy will be presented.

As a whole, our recent data will be compared to those obtained in other systems. Besides the fundamental importance of a novel mechanism of intracellular targeting, the results expected might be exploited in the future to develop an original system of complementation of pathogenic mutations in human mitochondrial DNA.

Oral Communication Abstract - 5.03

**COMPLETE SEQUENCE AND ANNOTATION OF THE CHLOROPLAST
GENOME OF GRAPE (*V. VINIFERA* L.) CV PINOT NOIR**

M. MOROLDO***, M. BARBARO*, S. SCALABRIN*, R. VELASCO**, M. MORGANTE*

*) Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Udine, Via delle Scienze 208, 33100 Udine

***) Istituto Agrario San Michele all'Adige, Via Mach 1, 38010 San Michele (TN)

chloroplast, Vitis vinifera, annotation, microsatellites

Chloroplast genomes of higher plants exhibit a remarkable level of similarity in sequence, organisation and gene order. They have small sizes (about 150 kbp) and contain about 120 genes; several microsatellites are found as well. Moreover, chloroplasts are mainly uniparentally unherited and their molecular lineages are not perturbed by recombination. For these reasons, they are a unique tool to study population genetics, to unveil the origin of polyploids and hybrids and to infer phylogenetic relationships.

In this work we present the first sequence of the chloroplast genome of grape (*Vitis vinifera* L.), together with its annotation for genes and microsatellites.

As a first step, a BAC clone containing the whole chloroplast genome was identified by analyzing through Blast search 27000 BAC ends from a BAC library of grape cv. Pinot Noir. The DNA extracted from this clone was then sheared by nebulization. Fragments in the size range of 1500-2500 bp were produced and then ligated into pCR-Script. The ligation mix was then used to transform *E. coli* strain DH10 β and about 3600 clones were picked up; about 6800 paired-ended sequences were obtained in this way. Further 500 sequences were obtained by Blast search on two genomic libraries of grape cv. Pinot Noir, in which cpDNA represents a contamination of nuclear DNA.

All the about 7300 paired-ended sequences were then assembled using Arachne2 (Whitehead Institute, MA) and 38 contigs were obtained. These contigs were furtherly processed using Cap3 (Huang and Madan, Genome Research 1999, 9(9): 868-877), reducing their number to 28. After this assembly the contigs were annotated using the web-based software DOGMA (<http://bugmaster.jgi-psf.org/dogma/>), which identifies genes by performing a Blast search against a custom database of genes from 16 complete chloroplast genomes of green plants.

Taking advantage of the high level of colinearity between chloroplast genomes and using *Nicotiana tabacum* as a reference, this first annotation step made it possible to know the relative order of the contigs between them. Moreover, this led to determine the position of the gaps, which were then closed by PCR amplification and sequencing.

Once the sequence was complete, it was annotated again for genes using DOGMA. 112 genes were identified, a number which is very close to the one usually found in green plants, and the gene order appeared to be the same known for plants like *Nicotiana tabacum*. A modified version of the software Sputnik (Morgante *et al.*, Nature Genetics 2002, 30(2): 194-200) was then run and 57 SSRs were found. Subsequently, a phylogenetic study was undertaken to better establish the position of *V. vinifera* among green plants, especially among the dycotyledonous.

To elucidate the patterns of evolution of microsatellites in cpDNA, the SSRs found in the plastid of grape were aligned with the syntenic loci of other species, allowing a direct comparison between the corresponding sequences to make inferences on mutations and homoplasy.

Oral Communication Abstract - 5.04

DEVELOPMENT AND APPLICATION OF THE PLASTID TRANSFORMATION TECHNOLOGY IN POTATO

D. GARGANO, V. VALKOV, P. LENZI, N. SCOTTI, S. GRILLO, T. CARDI

CNR-IGV, Institute of Plant Genetics, Via Università 133, 80055 Portici, Italy

potato, plastid transformation, desaturase, HPV, L1

Plastid transformation in higher plants allows precise transgene insertion by homologous recombination, high and stable gene expression and protein accumulation, gene coexpression in operons as well as gene containment through maternal inheritance of cytoplasmic organelles. So far, potato plastid transformation has been achieved mostly with reporter genes and the maximum efficiency was 10 times lower than that reported for tobacco. Herein we describe the development and application of the plastid transformation technology in potato for the expression of *S. commersonii* $\Delta 9$ desaturase and HPV16 L1 transgenes. $\Delta 9$ desaturase gene controls the insertion of double bonds in fatty acid chains; a high content of unsaturated fatty acids in plant cells can have a positive effect for tolerance to abiotic stresses as well as for nutritional purposes. VLPs made from the major capsid protein L1 of carcinogenic HPVs have been shown to induce protective immunity in animal models; the expression of the L1 gene in plant cells is an alternative approach for the development of a prophylactic vaccine.

The cv. Desiree was selected for transformation experiments based on preliminary regeneration data, while optimal conditions for gene delivery using the biolistic approach were set up using the nuclear vector pGUS-HYG (Craig et al. 2005).

In the process of improving the procedure for plastid transformation we tested three different regeneration protocols, using transformation vectors which target the inverted repeats or the large single copy of the chloroplast genome. In first experiments, one transplastomic shoot every 30 bombardments was regenerated on a medium containing 400 mg l⁻¹ spectinomycin, confirming the transformation efficiency reported in the literature. Later modifications of the procedures for the preparation of gold particles and for the tissue culture of explants allowed to obtain up to one transplastomic shoot every 2-5 bombardments.

Using the improved protocol, we have transferred the gene coding for $\Delta 9$ desaturase, isolated from the wild potato species *Solanum commersonii* to potato plastome. All transplastomic plants showed a high production of the desaturase transcript. Transplastomic plants with the L1 gene fused to a His6 tag also showed a strong signal after Northern analysis, but Western analysis carried out with anti-L1 antibodies did not detect the expected protein, although preliminary results with anti-His antibodies had showed the presence of two bands only in transplastomic plants.

Experiments with vectors containing different promoters and terminators are under way. Further, work in our laboratory is aiming to develop improved vectors for potato transformation. The complete sequence of the potato plastome has been obtained and will be used to isolate species-specific flanking and regulatory sequences. Finally, genomic approaches are being used to compare the expression of plastidial genes in chloroplasts and amyloplasts with the aim to identify regulatory sequences supporting high gene expression in non-green plastids.

Craig W, Gargano D, Scotti N, Nguyen TT, Lao NT, Kavanagh TA, Dix PJ, Cardi T 2005 Direct gene transfer in potato: a comparison of particle bombardment of leaf explants and PEG-mediated transformation of protoplasts. *Plant Cell Rep*, in press

Oral Communication Abstract - 5.05

ANALYSIS OF GENE EXPRESSION ASSOCIATED WITH CHLOROPLAST DEVELOPMENT AND COLD ACCLIMATION IN BARLEY ALBINA AND XANTHA MUTANTS

C. CAMPOLI*, S. CAFFARRI***, J. SVENSSON**, T. CLOSE**, R. BASSI***, L. CATTIVELLI*, C. CROSATTI*

*) CRA-Istituto Sperimentale per la Cerealicoltura, Via S. Protaso 302, 29017 Fiorenzuola d'Arda (PC), Italy

***) Department of Botany and Plant Sciences University of California, Riverside, CA, 92521-0124-USA

***) Université Aix-Marseille II, Laboratoire de Génétique et Biophysique des Plantes Département de Biologie 163, Avenue de Luminy- 13288 Marseille Cedex 09, France

oligo-array, barley, chloroplast development

We investigated the transcriptome of four albina and xantha barley mutants representing successive steps in chloroplast biogenesis and the corresponding wild type (WT) with the Affymetrix Barley 1 GeneChip to assess the variation of gene expression associated with chloroplast development.

Moving from *alb-e16* (the most extreme mutant) to *xan-b12* (the genotype closest to WT) the number of genes up- or down-regulated in the leaves decreased suggesting the progressive normalization of the transcriptome as chloroplast development proceed. The up and down regulated genes were converted to the arabidopsis homologous sequences for analysis with MAPMAN software to investigate the metabolic changes controlled by chloroplast.

Previously, we have shown that barley plants carrying mutations preventing chloroplast development are completely frost susceptible as well as impaired in the expression of several cold regulated genes. The availability of a barley microarray provides a new tool to describe the barley stress response and the role of the chloroplast in cold acclimation. 2735 genes were found more than 2 fold up- or down-regulated in cold hardened WT plants and their expression analysis allowed separation of the WT *cor* genes into separated classes (chloroplast dependent, chloroplast independent and chloroplast development dependent) showing about 70% of these genes were not cold regulated in any chloroplast mutants and these genes are independent to the CBF regulatory pathway. Among the genes regulated by cold only in WT about half were already expressed in the mutants at 20°C at levels similar to cold treated WT, and cold treatment did not change the expression of those genes in the mutants. Possibly a block in chloroplast development activated a signalling pathway that overlaps a pathway normally activated by low temperature.

The albina and xantha mutants are characterized by either the lack of carotenoids or a disruption of the assembly of photosynthetic membranes. Under both conditions extensive photo oxidative damage has to be expected in illuminated plants. To determine the involvement of photo oxidative stress on the cold gene expression we have compared the level of expression of such genes in mutant cold treated with the level of expression in WT treated with norfluorazon. The results suggest that a part of gene induction of albina and xantha mutants exposed to cold is do to photo oxidative stress.

Oral Communication Abstract - 5.06

FRATAXIN NULL MUTANTS OF ARABIDOPSIS ARE EMBRYO LETHALS

V. VAZZOLA, A. DE MARTINI, I. MURGIA, C. SOAVE

Department of Biology, University of Milan, Via Celoria 26, 20133 Milano, Italy - carlo.soave@unimi.it

mitochondria, embryogenesis, iron metabolism

Frataxin is a nuclear encoded protein targeted to the mitochondrial matrix. In humans, frataxin deficiency is associated with Friedreich's ataxia, a neurodegenerative and cardiac disorder characterized by accumulation of iron in the mitochondria and a diminished activity of various mitochondrial proteins, including aconitase. Yeast cells lacking frataxin show a complex respiratory deficient phenotype, defective in the maturation of mitochondrial Fe/S enzymes, hypersensitivity to oxidative stress, instability of mtDNA and defects in heme biosynthesis. It has been proposed that frataxin has ferroxidase activity and iron storage properties which may protect the mitochondria from iron toxicity, and that it also acts as a chaperone to donate iron to the proteins involved in the two major pathways of iron utilization, Fe/S cluster assembly and heme synthesis. Recently, an Arabidopsis gene (AtFH) highly similar to the human frataxin gene and possessing a mitochondrial targeting sequence has been described (Busi et al. 2004): AtFH is able to complement a yeast frataxin null mutant and in the plant is mainly expressed in flowers. We identified in the Salk collection two T-DNA insertions in the AtFH gene and characterized genetically the two mutants. Upon selfing heterozygous plants, we cannot recover in the progeny homozygote null seeds while homozygous wt and heterozygous seeds were in a ratio 1: 2 (as observed after PCR analysis). The ratio was consistent with lethality of the homozygous null genotypes during embryogenesis. Accordingly we analyze the pattern of embryo development in siliques segregating homozygous null embryos: an early arrest at the 8-16 cells stage was consistently observed.

Busi M. et al. FEBS Letters 576, 141-144, 2004.

Oral Communication Abstract - 5.07

**A MUTATION IN THE PENTATRICOPEPTIDE REPEAT-CONTAINING GENE
EMPTY PERICARP-4 AFFECTS SEED DEVELOPMENT IN MAIZE**

M. DAL PRÀ*, A. GIULINI*, G. GAVAZZI*, J. F. GUTIÉRREZ-MARCOS**, L. M. COSTA**,
H. G. DICKINSON**, S. CORDELIER***, O. SELLAM***, C. TATOUT***, W. PAUL***,
P. PEREZ***, G. CONSONNI*

*) Dipartimento di Produzione Vegetale, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

**) Department of Plant Sciences, Oxford University, South Parks Road, Oxford, OX1 3RB, United Kingdom

***) Biogemma, 24 Avenue des Landais 63 170 Aubière, France

pentatricopeptide repeat protein, seed development, endosperm development, maize, emp4

The pentatricopeptide repeat (PPR) family represents one of the largest gene family in plants, with more than 440 members annotated in *Arabidopsis*. Studies conducted on some individual members of the PPR family indicate that these proteins are localized to organelles where they participate in gene expression. A similar picture emerges from a genomic study conducted in *Arabidopsis* which show that PPR proteins are localized in mitochondria and chloroplasts. Thus PPR proteins are thought to be important for transcriptional and postranscriptional processes in organelles. Mutant analyses have shown that *Arabidopsis* PPR proteins retain an essential, non-redundant role in embryogenesis. We will present the characterization of *emp4*, a mutant in a maize PPR gene, which confers a seed lethal phenotype, as a result of severely impaired endosperm development. The *emp4-1* mutation was identified in an active *Mutator* population and co-segregation analysis showed that it was due to a *Mutator3* element insertion. Evidence for *emp4* molecular isolation was provided by a reverse genetic strategy that resulted in the isolation of four additional *emp4* alleles. *emp4* defines a novel single copy maize *emp* gene, located on the long arm of chromosome 1. Its product contains nine repeats of the 35-aa pentatricopeptide motif and it exhibits a highly conserved structure with two already identified *Arabidopsis* PPR proteins as well as with the product of a unique rice sequence. The PPR product of *emp4* is required for the correct differentiation of endosperm domains and in particular for basal endosperm transfer layer (BETL) differentiation. A GFP fusion construct with the entire EMP4 protein localizes to mitochondria, suggesting a possible role for the protein in these organelles.

ZEOLIN EXPRESSION IN TOBACCO CHLOROPLAST AND ALFALFA ENDOPLASMIC RETICULUM

M. BELLUCCI*, F. DE MARCHIS*, H. DANIELL**, A. VITALE***, S. ARCIONI*

*) Institute of Plant Genetics - Research Division of Perugia - CNR, Via della Madonna Alta 130, 06128 Perugia, Italy

**) Department of Molecular Biology and Microbiology - University of Central Florida, Biomolecular Science, Orlando, FL 32816-2364, USA

***) Institute of Agricultural Biology and Biotechnology, Research Division of Mi-Bassini - CNR, Via Bassini 15, 20133 Milano, Italy

zein, phaseolin, chloroplast, Nicotiana tabacum, Medicago sativa

The major seed storage proteins of *Zea mais* and *Phaseolus vulgaris*, zein and phaseolin, accumulate in the endoplasmic reticulum and in storage vacuoles, respectively. Zeolin polypeptide, composed of phaseolin and the first 89 amino acids of γ -zein, shown to form endoplasmic reticulum-located protein bodies in transgenic tobacco leaves (Mainieri et al., 2004, Plant Physiology 136: 3447-3456). Fusion with the gamma-zein domain which has been utilised to construct zeolin, could be a useful strategy to accumulate target proteins of pharmacological interest in transgenic plants. In order to verify if zeolin accumulates also in other plant species, alfalfa (*Medicago sativa* L.) plants have been transformed with the zeolin gene and the protein has been detected by western blot in alfalfa leaves. Moreover, zeolin forms endoplasmic reticulum-located protein bodies even in transgenic alfalfa leaves. Previously, it has been shown that zeolin is highly stable when accumulated in the endoplasmic reticulum. To investigate if the ability of zeolin to accumulate to very high levels was limited to this sub-cellular compartment, we inserted the zeolin gene in the tobacco chloroplast DNA. The zeolin gene was cloned in a plastid expression cassette into the chloroplast transformation vector pLD-CtV which targets the transgene cassette to the 16S-*trnI*/*trnA*-23S region of the chloroplast genome. A comparison between the two kinds of zeolin sub-cellular localization by western blot analysis shown that zeolin accumulated in the chloroplast to lower levels than in the endoplasmic reticulum. The considerable presence of phaseolin and gamma-zein polypeptides with respect to intact zeolin, suggests that in the chloroplast a high amount of zeolin is fragmented. No zeolin protein bodies were visualised in the chloroplast by electron microscopy.

Poster Abstract - 5.09

FUNCTIONAL ANALYSIS OF XANTHOPHYLLS IN *ARABIDOPSIS THALIANA* L.

A. FIORE*, C. LICO*, R. TOMMASI*, L. DALL'OSTO**, R. BASSI*****, M. HAVAUX***, G. GIULIANO*

*) Ente per le Nuove tecnologie, l'Energia e l'Ambiente (ENEA), Biotechnology Unit, Casaccia Research Centre, PO Box 2400, Roma 00100AD, Italy

**) Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15, I-37134 Verona, Italy

***) CEA/Cadarache, DSV, DEVM, Laboratoire de Génétique et Biophysique des Plantes, UMR 163 CEA-CNRS-Univ. Méditerranée, 163 avenue de Luminy, F-13288 Marseille cedex 09, France

Plant carotenoids are 40-carbon isoprenoid compounds involved in many physiological and biochemical functions, such as photosynthetic light harvesting and photoprotection from excess light energy. Xanthophylls, oxygenated forms of carotenes, are accessory pigments in the light-harvesting antennae, involved in the energy transfer to the chlorophylls and in non-photochemical quenching (NPQ). To elucidate the *in vivo* functions of xanthophylls in photosystem biogenesis and their relation with oxidative stresses, we identified mutants in the β -carotene hydroxylase 1 and 2 genes, the *e*-cyclase gene and the *e*-hydroxylase gene and its paralog. Pyramiding of the mutants is being used to construct *Arabidopsis* plants synthesizing only one, or few, xanthophylls such as the *chl1chl2lut2* triple mutant which shows a lower content of neoxanthin and violaxanthin, lacking lutein completely, or the *lut2npq2* double mutant, where all leaf xanthophylls are replaced by zeaxanthin. Further studies are in progress in order to characterize these mutants at the phenotypic, biochemical and molecular levels.

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TRANSPLASTOMIC TOBACCO AS BIOFACTORY FOR THE PRODUCTION OF HIV-1 GAG ANTIGEN

F. ALAGNA*, N. SCOTTI*, S. GRILLO*, M.L. TORNESELLO**, L. BUONAGURO**,
F. BUONAGURO**, T. CARDI*

*) CNR-IGV, Institute of Plant Genetics, Res. Div. Portici, Portici (NA), Italy - nscotti@unina.it

***) Viral Oncology, Ist. Naz. Tumori "Fond. G. Pascale", Napoli, Italy

plastid transformation, HIV, human vaccine

The development of an effective and safe vaccine strategy for HIV virus represents a crucial goal for both industrialized and developing countries. So far, different candidate HIV vaccine strategies have been developed. A promising approach is based on the property of the capsid protein Gag (Pr55) to auto-assemble and form Virus-Like Particles (VLPs) displaying several viral epitopes on the surface.

Recently, there is an increasing interest towards the use of plants as biofactories to produce industrial products and pharmaceuticals. In comparison with other production systems, plants show a lower cost, a higher yield, the lack of pathogens and/or toxins dangerous to human health, a higher flexibility towards changes in the product request. However, the use of transgenic nuclear plants as bio-factories generally resulted in low expression levels of antigenic proteins. To overcome these limits, we are attempting to express the capsid protein Gag (Pr55) in the plastidial genome to test the feasibility of using this technology for the production of plant-derived HIV antigen. The transformation of the plastidial genome generally shows high level of gene expression and absence of gene silencing, the possibility to coexpress several genes in an operon under the control of a single promoter, the precise integration of transgenes by homologous recombination, the containment of transgenes and gene products.

The *gag* gene from HIV-1 was cloned, with or without an N-terminal His6-tag, under the control of the strong promoter Pr_{rrn} and the 5'-UTR of the T7 phage gene 10 in vectors designed for the integration of transgenes in either the inverted repeats (IR) or the large single copy region (LSC) of the plastidial genome (courtesy of Dr. P. Maliga, USA, and Dr. R. Bock, Germany). Recombinant vectors were used for biolistic transformation of *Nicotiana tabacum* (cv Petite Havana) plants.

So far, transplastomic plants were obtained with the *gag* construct without the N-terminal His6-tag. The characterization of spectinomycin and streptomycin-resistant plants by PCR and Southern analyses demonstrated the correct integration of the transgene and a high degree of homoplasmy. Northern analysis showed a high level of *gag* gene expression, whereas the analysis of protein expression with polyclonal human antibodies from blood serum of AIDS patients revealed aspecific bands both in transplastomic plants and untransformed control. Further experiments with recombinant HIV-1-p24 antiserum are under way.

Poster Abstract - 5.11

THE PLASTID GENOME OF POTATO (*SOLANUM TUBEROSUM* CV DÉsirÉE): COMPLETE NUCLEOTIDE SEQUENCE AND GENE ORGANIZATION

D. GARGANO*, A. VEZZI**, N. SCOTTI*, A. BILARDI**, G. VALLE**, S. GRILLO*, T. CARDI*

*) CNR-IGV, Institute of Plant Genetics, Via Università 133, 80055 Portici, Italy

***) CRIBI Biotechnology Centre and Department of Biology, Univ. of Padova, Via Bassi 58/B, 35131 Padova, Italy

Chloroplasts are organelles which contain their own genetic systems. The complete nucleotide sequence of chloroplast genomes was first reported in tobacco and liverwort in 1986. Since then, the plastome sequences of a number of higher plants and algae have been determined. Herein we report the complete nucleotide sequence (155298 bp) and gene organization of potato (*Solanum tuberosum* cv. Désirée) chloroplast DNA. It contains two copies of identical 25594 bp inverted repeats, which are separated by a 85737 bp large single copy and a 18373 bp small single copy regions.

Potato cpDNA was extracted after leaf chloroplast purification onto a sucrose gradient. A recombinant library was obtained by cloning *Bam*HI, *Xho*I, *Pst*I and *Sal*I cpDNA fragments either in pUC19 or pGEM vectors. Additionally, chloroplast DNA was processed by mechanical shearing to produce fragments 1.5-3.0 kbp in size. Fragments in the size range of 1.5-2.0 kbp and 2.0-2.5 kbp were extracted from gel and ligated into pZero vector. Recombinant colonies were obtained with both fragment size ranges, but only those derived from the 1.5-2.0 kbp library were processed. Twelve contigs were initially obtained by assembling 1536 sequences with the Phred/Phrap/Consed software (P. Green, B. Ewing, D. Gordon, University of Washington, USA). After adding about 70 more sequences during the finishing phase, the number of contigs decreased to four. The remain four gaps were closed by PCR. The complete nucleotide sequence was annotated using DOGMA software (Wyman et al. 2004) and 114 genes and *ycfs* were found. Further, about half of the tobacco-specific open reading frames seem to be present also in the potato plastome as well as the two *ori* and *ars* sequences. Differences between the two species in the J_{LA} and J_{LB} regions were confirmed. A total of 306 indels is responsible for the difference in length between the plastomes of the two species. The largest insertion (543 bp) and deletion (430 bp) were found in the LSC in the intergenic regions between *ycf4* and *cemA*, and *trnE* and *trnT*, respectively. Based on indel frequency and percent identity, the nucleotide sequence in the inverted repeats was more conserved than in the two single copy regions.

This sequence will be useful to understand evolutionary divergence in the *Solanaceae* family and to construct specific vectors for potato plastid transformation.

Poster Abstract - 5.12

BIPARENTAL PLASTID INHERITANCE IN *MEDICAGO SATIVA* REASSESSED USING PLASTOME POLYMORPHISMS

D. ROSELLINI, N. FERRADINI, M. CARCIOFI, S. CAPOMACCIO, F. VERONESI

Dipartimento di Biologia Vegetale Biotecnologie Agroambientali e Zootecniche, Università degli Studi di Perugia – roselli@unipg.it

alfalfa, chloroplast, microsatellites, Plastome inheritance

Medicago sativa is known to have bi-parental plastid inheritance with paternal bias, as demonstrated using chloroplast (CP) mutations. With the final objective to investigate if this peculiar type of plastid inheritance is also present in other *Medicago* species, we are developing plastid DNA markers suitable for this purpose. Two unrelated *M. sativa* genotypes, named CLA and SAL, were reciprocally crossed and evaluated for 10 published CP microsatellites that, surprisingly, were all monomorphic. Two previously described CP DNA hypervariable regions (Skinner 2000) were then amplified and one of them (named *HindIII* region) was polymorphic giving an amplicon of about 500bp in CLA and 700 in SAL.

Progeny plants from the reciprocal crosses were so far screened with the *HindIII* polymorphism. Of 94 SAL x CLA progenies, 87 (92.6%) had the paternal marker, 5 (5.3%) had the maternal marker, and two was heteroplasmic, displaying both markers. The genomic DNA was re-extracted from these two individuals and heteroplasmy was confirmed. One plant had the paternal band plus a shorter amplicon of about 400 bp, probably the result of a replication-related deletion that has previously been documented for this region containing direct repeats. Of 76 CLAxSAL progenies, 61 (80.3%) had the paternal marker, 14 (18.4%) had the maternal marker, and one, displaying both markers. The alfalfa CP inheritance pattern is therefore confirmed in this cross.

The second hypervariable region (named *HaeII*) appeared to be monomorphic after agarose gel electrophoresis, giving an amplicon of about 1300 bp. Because the sequence of this region is not published, we sequenced it. The high polymorphism previously found in this region among alfalfa germplasms is probably related to the presence of an AT-rich direct repeat of about 64 bp present in 5 copies. We found a 5 bp and two single base deletions in SAL, that can be used to design primers for an allele-specific PCR test.

The PCR-based markers used in this preliminary experiment will be tested in *Medicago truncatula*, a model species in which, to our knowledge, plastid inheritance has not yet been investigated. The sequence of most of the primers developed for alfalfa CP markers was found in the *M. truncatula* published plastome sequence.

Reference

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MOLECULAR CHARACTERIZATION OF LHCb1 ISOFORMS IN SPINACH AND THEIR RESPONSE TO HIGH LIGHT EXPOSURE

G. REA*, A. DISTASO**, L. ZOLLA***, R. D'OVIDIO****, R. GALLERANI**, L.R CECI*****

*) Institute of Crystallography, CNR, Monterotondo Stazione, Rome, Italy

***) Department of Biochemistry and Molecular Biology, University of Bari, Italy

****) Department of Environmental Sciences, University of Tuscia, Viterbo, Italy

*****) Department of Agrobiological and Agrochemistry, University of Tuscia, Viterbo, Italy

*****) Institute for Biomembranes and Bioenergetic, CNR, Trani, Bari, Italy

light harvesting complex, high light stress, Spinacia oleracea

The plant light-harvesting complex of photosystem II (LHC-II) collects and transmits solar energy for photosynthesis in chloroplast membranes and has essential roles in regulation of photosynthesis and in photoprotection. Native LHC-II isolated from plant tissue consist of three isoforms, LHCb1, LHCb2 and LHCb3, which form homo- and heterotrimers. Other LHC proteins are present in plants, which are also known as CAB proteins for their capacity to bind chlorophyll (Chl) *a* and Chl *b*. There are 10 known CAB proteins in higher plants: LHCb1-6 associated with photosystem-II, and LHCa1-4 associated with photosystem-I. In photosystem II, LHCb1-3 form a mobile and external complex, LHCb4-6 are more closely associated with the core of the photosystem and are not mobile. Various studies carried out on different species, indicate the presence of different isoforms for the single polypeptides antenna of photosystem-II and photosystem-I. Currently, the role of different isoforms is poorly understood.

Although the isolation of spinach PS-II has been reported since 1981, and numerous biochemical and biophysical studies have been carried out on it, a complete description of genes coding for members of the spinach supercomplex, has not been reported yet. Currently only the genes coding for Lhcb1 and Lhcb6 polypeptides have been sequenced.

By taking advantage of the published sequences of *Lhcb1* gene in several plants, we have designed a couple of degenerated oligonucleotides which could amplify different *Lhcb1* isoforms occurring in the spinach genome. Sequencing of amplified regions allowed to identify three sequences encoding polypeptides which perfectly match the partial amino acid sequences of spinach Lhcb1 isoforms previously determined. RACE and Real-Time RT PCR analysis were carried out in order to get full sequences of cDNAs and to understand the possible involvement of these isoforms in plant adaptation to different light conditions. Obtained results will be presented and discussed here.

THE DIFFERENTIAL EXPRESSED BRANCHED-CHAIN AMINOTRANSFERASE (HvBCAT-1) FROM BARLEY IS CAPABLE TO COMPLEMENT YEAST DEFICIENCY

M. MALATRASI***, M. CORRADI*, J. T. SVENSSON**, T. J. CLOSE**, M. GULLI*, N. MARMIROLI*

*) Dipartimento di Scienze Ambientali, Sez. Genetica e Biotecnologie Ambientali, Parco Area delle Scienze, Università di Parma, 43100 Parma, Italy

***) Department of Botany and Plant Sciences, University of California, Riverside, CA 92521-0124, USA

branched-chain amino acid aminotransferase, abiotic stress, barley, DDRT-PCR, functional complementation

The BCAs (branched-chain amino acids) are essential amino acids which plants are able to “de novo” synthesize. Very little is known about the last step of the catabolic/anabolic pathway catalyzed by the branched-chain amino acid aminotransferases (BCATs). Analysis of gene expression in barley seedling shoots by Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT-PCR) led to the isolation of several drought-, cold- and ABA-induced cDNA short sequences. One of these (DD12) corresponds the HvBCAT-1 gene, which belongs to the BCAT gene family in barley. The structure of HvBCAT-1 has been elucidated: the coding region is interrupted by six introns and contains a predicted mitochondrial transit peptide, the gene has also been mapped on chromosome 4H. A comparative analysis with the homologous genes from rice and *Arabidopsis* has been performed in order to identify conserved structural patterns. Complementation of a yeast (*Saccharomyces cerevisiae*) double knockout strain revealed that HvBCAT-1 can function as the mitochondrial (catabolic) BCATs *in vivo*. The transcriptional levels of HvBCAT-1, analyzed by Northern hybridization, by qualitative and quantitative RT-PCR and by microarray, under several conditions resulted increased in relation to drought condition. Tentatively, (as the first enzyme in BCA catabolic pathway) HvBCAT-1 might have a role in the increased degradation of BCA, previously discussed as a detoxification mechanism that maintains the pool of free branched-chain amino acid at low and not toxic levels in stress condition.

Poster Abstract - 5.15

ANALYSIS OF TRANSCRIPTOME IN POTATO AMYLOPLASTS AND CHLOROPLASTS

V. VALKOV*, N. SCOTTI*, D. MACLEAN**, J. GRAY**, S. GRILLO*, T. CARDI*

*) Institute of Plant Genetics – CNR, Research Division Portici, Via Università 133, 80055 Portici, Italy

***) Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK

plastid transcription, Solanum tuberosum, amyloplasts, microarrays

Expression of plastidial genes is a complex process involving RNA processing and plastid-nuclear interaction. We are studying transcription of plastid genes during different developmental stages of tubers in order to identify genes that are actively transcribed and whose transcripts accumulate in amyloplasts of potato tubers.

Transcription of the plastid genes were investigated by microarray analysis. Glass microarray slides carrying spotted PCR amplified fragments from all tobacco plastid genes were hybridized with Cy3 and Cy5 labelled cDNA, generated from total RNA extracted from potato tubers of two different sizes and from leaves. Six independent microarrays hybridizations were performed per each experiment (big tubers vs. leaves, small tubers vs. leaves and small tubers vs. big tubers) including two dye swaps. The Cy3 and Cy5 fluorescent images were quantified, corrected for background fluorescence and the average transcript ratios were calculated for each gene. Results from microarrays indicate that transcription of all genes in tubers (either big or small) was down regulated comparing with leaves. However, in tubers most of the genes for the genetic apparatus (e.g. *rpl* and *rps*) were more expressed than photosynthetic genes. Genes with the highest fluorescence ratio tubers vs. leaves included *rpoC1* and *rpoC2*, *rps4*, *rps14*, *rps16* and some hypothetical chloroplast reading frames as *ycf6* and *ycf9*.

End-labeled RNA hybridization was used in order to confirm results obtained from the microarray analysis. Filters carrying 25 PCR amplified potato plastid genes selected on the basis of arrays were probed with 5'-end labeled RNA from leaves and potato tubers. Resulting signals were quantified after subtracting background hybridization to the negative control - vector pUC18. In general, the results from these experiments were correlated with the arrays data, showing, from one side, no significant difference in transcription level during tuber development, and from the other, again genes as *rpoC1*, *rpoC2*, *ycf6*, *rps16* displaying relatively high transcript accumulation in tubers compared to leaves. Based on transcript accumulation data, *rbcL* and rRNA genes were the highest expressed genes both in leaves and tubers.

Run-on and Northern blot analyses are under way in order to study transcription activity of the plastid genes and to confirm the results of steady state analyses.

GENETIC TRACEABILITY OF CLAM SPECIES USING MITOCHONDRIAL MARKERS

F. MARETTO*, E. REFFO*, G. BARCACCIA**, R. MANTOVANI*

*) Department of Animal Science, Agriculture Faculty, University of Padova. Agripolis, V.le dell'Università 16, 35020 Legnaro, Padova

***) Department of Environmental Agronomy and Crop Science, Agriculture Faculty, University of Padova. Agripolis, V.le dell'Università 16, 35020 Legnaro, Padova

CAPS markers, mtDNA polymorphisms, Meretrix spp., Ruditapes spp., Chamelea gallina

Genetic differentiation of the clam species *Meretrix meretrix*, *Meretrix lusoria*, *Ruditapes decussatus*, *Ruditapes philippinarum* and *Chamelea gallina* is very important for the authentication and the correct labelling of sea food products. Morphological criteria such as shell characteristics (shape, color and striation) as well as siphon shape and location allow to distinguish among these species when they are sold alive. Nevertheless, they are usually commercialised frozen and mixed with other ingredients. Since the shell is removed there is a real possibility that these species can be accidentally or fraudulently substituted. Due to their difference in prices, taste and flesh texture, nonmorphological methods are then necessary for the authentication of bivalves in order to assure consumers about the identity and quality of food products purchased. Moreover, it is a legal requirement for their commercialisation to show on the label the scientific and commercial name of the species, the production method and the capture zone. Finally, an additional important requirement is that companies (for instance, import/export companies) in recent years ask to safeguard themselves from potential swindles or improper commercialisation of sea food products.

This study is part of the project 'Study of new analytical and biotechnological methods for species control and traceability of different animal products (bovine, ovine and fish chain)', supported by Regione Veneto and CNR, in collaboration with private companies. The aim in the fish chain is to develop new molecular marker systems and reliable mitochondrial DNA-based assays for the identification of clam species. Clams imported from different sea areas of the world were kindly provided by Rivamar (Taglio di Po, Rovigo). Initially CAPS (*Cleaved Amplified Polymorphic Sequence*) markers were developed mainly on the 16s mitochondrial gene. New specific primer pairs were designed from the multiple alignment of known mitochondrial sequences of *Ruditapes decussatus* and *Ruditapes philippinarum* as well as other different organisms. Amplicons were sequenced to confirm that the amplified products were from the target 16s rRNA region and to design suitable restriction sites. Different combinations of restriction enzymes were used to distinguish among these species. CAPS markers proved to be effective, cost and time saving, and offered several advantages over protein analysis (i.e. electrophoretic, chromatographic and immunological techniques). DNA is less affected by processing as it is more stable and it is also present in all cells of a given organism, enabling to obtain the same information from any tissue. Single-locus polymorphisms developed through the analysis of genomic DNA with AFLP (*Amplified Fragment Length Polymorphism*) markers as well as SNP (*Single Nucleotide Polymorphism*) markers designed on known genic DNA sequences will be also investigated in order to set up a molecular assay suitable to distinguish clam species in a faster and easier way.

PLANT UNCOUPLING MITOCHONDRIAL PROTEIN (PUMP): AN ENERGY DISSIPATING SYSTEM ACTIVATED IN RESPONSE TO OSMOTIC STRESS IN DURUM WHEAT

D.TRONO*, M. SOCCIO* **, A.M. MASTRANGELO*, N. DI FONZO*, L. CATTIVELLI*,
Z. FLAGELLA**, D. PASTORE**

*) Istituto Sperimentale per la Cerealicoltura - CRA, SS 16 Km 675, 71100 Foggia, Italy -
trono@iscfoggia.it

***) Dipartimento di Scienze Agroambientali, Chimica e Difesa Vegetale, Facoltà di Agraria, Università
di Foggia, Via Napoli 25, 71100 Foggia, Italy

durum wheat mitochondria, uncoupling protein, salt stress, osmotic stress

Plant uncoupling mitochondrial protein (PUMP) represents an energy dissipating system able to uncouple mitochondria through a free fatty acid (FFA)-activated H^+ cycling process and to control mitochondrial production of reactive oxygen species (ROS). Whereas it is generally agreed that PUMP acts as a defense mechanism against oxidative stress, data concerning its involvement in plant response to environmental stresses, which lead to oxidative stress, are still lacking.

In order to shed some light into this aspect, an investigation was carried out concerning PUMP functioning in durum wheat mitochondria purified from etiolated early seedlings submitted to moderate and severe salt (NaCl) and osmotic (mannitol) stress.

Results on “proton leak” kinetics of mitochondria, demonstrated an increase in PUMP functioning, expressed as proton conductance (“proton leak”/ $\Delta\Psi$) at 100 mV, that was stress-intensity-dependent and more evident under salt than under osmotic stress, but this result did not allow to establish whether PUMP functioning increase was due to protein activation and/or to an increase in protein amount.

To investigate about this point, a new method, based exclusively on $\Delta\Psi$ measurements, was properly developed to evaluate both *i*) PUMP functioning due to the presence of endogenous FFA and *ii*) maximal PUMP activity, which may estimate protein amount, induced by externally added FFA to succinate-energized mitochondria. Values concerning PUMP functioning due to the presence of endogenous FFA highly correlated with values obtained as proton conductance at 100 mV, thus showing the suitability of the new method. As regards maximal PUMP activity, $\Delta\Psi$ measurements showed no or little (below 30%) increase, thus suggesting that protein content under stress conditions remains almost unchanged. Consistently, no increase in the expression level of plant UCP-related genes under stress conditions was observed in terms of transcript amount. The above results suggest that the increase in PUMP functioning observed under stress conditions is due to protein activation by modulators rather than to an increase in protein amount. In line with previous findings showing PUMP activation by ROS and by the increase in FFA availability, we found that under all stress conditions *i*) an increase in mitochondrial ROS production and FFA content occurred, with the latter being due to an increase in mitochondrial phospholipase A₂ activity (PLA₂), whose existence was demonstrated here for the first time; *ii*) PUMP activation was prevented by catalase plus superoxide dismutase and by bovine serum albumin, which remove ROS and FFA, respectively; *iii*) once activated, PUMP may lower ROS production, probably according to a feed-back mechanism.

In conclusion, an increase of ROS production, PLA₂ activity and FFA content occurs in mitochondria from early seedlings suffering hyperosmotic stress, leading to activation of PUMP, that in turn, may act as a defense mechanism by controlling further large scale ROS production. On the contrary, transcript levels of plant UCP-related genes and maximal PUMP activity remain unchanged.