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Oral Communication Abstract – 2B.01

UNFOLDED PLASTID PRECURSORS IN THE CYTOSOL: TARGETING VS DEGRADATION BY QUALITY CONTROL

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A large number of nuclear encoded proteins are targeted to plastids posttranslationally as unfolded precursors. Thus, navigation of them through the cytoplasm is intrinsically dangerous to the cell viability because their accumulation in cytosol due to faulty import process can result in formation of cytotoxic and life threatening non-specific aggregates. Therefore cells must have a mechanism to monitor targeting of precursors to plastids and prevent cytosolic accumulation of precusors. Recently, we demonstrated Arabidopsis Hsp70-4, a member of heat shock protein 70 and its interacting E3 ligase AtCHIP play critical role in this process. Hsp70-4 and AtCHIP causes degradation of unimported or import defective precursors. The degradation was mediated by the ubiquitination/proteasome system. Hsp70-4 specifically recongizes and binds to sequence motifs present in the transit peptide of RbcS in vitro and in vivo and induces ubiquitination of unimported precursors. In *ppi2* mutants with a T-DNA inertion in *Toc159*, encoding the major import receptor, transcription of Hsp70-4 and AtCHIP was elevated. In ppi2 plants, endogenous RbcS and Cab precursors were degraded by the Hsp70-4 and AtCHIP-mediated UPS. In addition, in this pathway, BAG isoforms also are involved in positively or negatively depending on individual isoforms.

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Oral Communication Abstract – 2B.02

DISSECTING THE ENDOMEMBRANE NETWORK VIA CHEMICAL GENOMICS

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Although it is known that proteins are delivered to and recycled from the plasma membrane (PM) via endosomes, the nature of the compartments and pathways responsible for cargo and vesicle sorting and cellular signaling is poorly understood. Such highly dynamic processes are not easily approached genetically. To define and dissect specific recycling pathways, rapid-acting chemical affecters of proteins involved in vesicle trafficking, especially through endosomes, would be invaluable. Thus, we identified chemicals affecting essential steps in PM/endosome trafficking by utilizing the intensely localized PM transport at the tips of germinating tobacco pollen tubes. We screened diverse chemical libraries for those that interfered with pollen germination and tip growth. We found that many also had effects in Arabidopsis roots for which there are several well-characterized marker proteins that cycle to and from the plasma membrane. Recent results from our screens will be discussed.

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Oral Communication Abstract – 2B.03

INTERCHAIN DISULFIDE BOND FORMATION IN THE ASSSEMBLY OF HIGH- AND LOW-MOLECULAR-WEIGHT GLUTENIN SUBUNITS

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Tritucum aestivum, Triticum durum, High-Molecular-Weight Glutenin Subunit, Seed Storage Proteins, Gluten Proteins

Wheat seeds are one of the most important protein and energy sources for human nutrition. In addition, wheat seed proteins have unique functional characteristics that make wheat flour suitable for bread and pasta making. One major group of gluten proteins is constituted by the polymeric glutenins, consisting of Low-Molecular-Weight (LMW) subunits, High-Molecular-Weight (HMW) subunits, and “aggregated” gliadins. Interchain disulfide bonds play a crucial role in stabilizing the glutenin polymer but the organization of the different subunits remains largely unknown. To gain insight into the mechanisms controlling the assembly of glutenin polymers, we have expressed glutenin subunits, individually or in combination, in tobacco protoplasts. Using this system we have compared the assembly of two HMW subunits, Bx7 from *T. aestivum* cv Glenlea and Bx20 from *T. durum* cv Bidi17. The over-expression of Bx7 is associated with good pasta-making phenotype, while Bx20 has an opposite effect. The two proteins have similar amino acid sequence, but differ in the number of cysteine residue. The Bx20 precursor is a large protein of 795 aminoacids, and contains two cysteine residues, one in the N-terminal and one in the C-terminal non-repetitive domains (C31 and C783). The Bx7 subunit has 795 aminoacids, with four cysteines, three in the N-terminal (C31, C38 and C53), and one (C783) in the C-terminal domain. The role of individual cysteine residues in the formation of intermolecular disulfide bonds was studied through mutagenesis and substitution with the non polar aminoacid alanine. Bx20 mutants containing a single cysteine residue could still form homodimers, indicating that the protein can assemble via C31-C31 and C783-C783 interchain disulfide bonds. In addition, the two single cysteine mutants were able to dimerize with a LMW-GS, indicating that both cysteine residues in the Bx20 proteins can be involved in the interaction with this second class of gluten proteins. In the Bx7 protein, the C783A mutation was sufficient to block polymer formation, the mutant protein being recovered in monomeric and dimeric form. This indicates that the N-terminal domain of the protein, although it contains three cysteine residues, cannot by itself support polymerization, possibly because of the presence of an intrachain disulfide bond and/or because of a steric hindrance phenomenon. These results raise the possibility that, notwithstanding the different number of cysteine residues, both Bx20 and Bx7 subunits can only form linear, rather than branched polymers.

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Oral Communication Abstract – 2B.04

AN *ARABIDOPSIS* TAIL-ANCHORED PROTEIN INVOLVED IN ORGANELLE FISSION UNVEILS A DYNAMIC NETWORK OF ENDOMEMBRANE STRUCTURES

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Mitochondria, peroxisomes, chloroplasts, organelle fission, inter-organelle connections

In plant cells, metabolic connections, functional interactions and signaling cross-talk are established among peroxisomes, mitochondria and plastids. However, until now it is not clear how this directional trafficking of metabolite/signalling molecule is achieved, and whether physical connections are established among peroxisomes, mitochondria and plastids. In *Arabidopsis*, peroxisomes share with mitochondria and chloroplasts some molecular components of their fission machinery (e.g. FIS1A and DRP5). In particular, the tail-anchored FISSION1A (FIS1A) protein is localized on the membranes of peroxisomes and mitochondria and it is implicated in peroxisomal and mitochondrial fission. Interestingly, there are some indications that FIS1A is also localized on chloroplasts. The DYNAMIN RELATED PROTEIN5 (DRP5) is instead involved in the fission of peroxisomes and chloroplasts. DRP5 interact *in vivo* with FIS1A on peroxisomes, while so far their interaction on chloroplasts has not been investigated.

In this work, we analyzed the expression pattern and the subcellular localization of FIS1A on different organelles during plant development, generating stable transgenic plants. Our data indicate that the sub-cellular localization of FIS1A is not restricted to the compartments so far described, unveiling the existence of connections among the different organelles mediated by tubular protrusions extending from them. We analyzed the motility of these tubular protrusions *in vivo* and the trafficking of FIS1A inside this network, investigating whether there was a physical continuity among the different organelles. In order to investigate *in vivo* the mechanism involved in the multiple subcellular targeting of FIS1A, we obtained stable transgenic plants expressing truncated FIS1A proteins. Moreover, in order to gain inside the role of FIS1A in the organelle remodeling, we tested and localized the interactions *in vivo* of FIS1A with some cytosolic proteins implicated in organelle fission (e.g. DRP5).

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Oral Communication Abstract – 2B.05

ONLY A MINORITY OF INTEGRAL MEMBRANE PROTEINS WITH COMPLEX N-GLYCANS RESIDE ON THE TONOPLAST

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Complex N-glycans, membrane proteins, tonoplast biogenesis, N-glycoproteome

About one third of the secretory proteins of the cell are N-glycosylated and their glycans are very often modified when proteins travel throught the Golgi complex. Tonoplast proteins can reach the membrane either passing or bypassing the Golgi. To investigate on the extent of Golgi involvement in the traffic of tonoplast proteins, we therefore analyzed the subcellular distrubution of Arabidopsis proteins with complex glycans. Plant N-glycans can be modified by Golgi enzymes, acquiring Golgi-specific complex sugars (by addition of b1,2 Xylose, a1,3 and a1,4 Fucose). Intact vacuoles were purified from Arabidopsis leaves. Peripheral membrane proteins were removed before sub-fractionation by centrifugation to separate tonoplast microsomes form the vacuolar content. As a control, total microsomes and soluble proteins were purifed from protoplasts. The samples were subjected to SDS-PAGE and protein blot using antisera against organelle markers (BiP, GRP96, gTIP) to verify the purity of fractions or antiserum against plant complex glycans, which recognizes plant Golgi-modified glycans containing Fuc or Xyl. Total microsomes were also prepared from Arabidopsis leaves and fractionated by sucrose density gradient at the equilibrium. The origin of microsomes in the fractions were determined using antisera against marker proteins from plasma membrane (PIP2) or tonoplast (TIP). Our results demonstrate that: i) the bulk of the proteome with complex N-glycans is constituted by soluble proteins; ii) among membrane proteins, the majority is at the plasma membrane, while only a minor proportion of tonoplast proteins has complex glycans. Analysis using concanavalin A, which binds high-mannose (unmodified) N-glycans is under way to determine whether the results reflect a minor contribution of N-glycoproteins to the tonoplast proteome or a major tonoplast traffic route bypassing the Golgi complex. The biological implication of these data will be discussed.

Study supported by the EU Marie Curie Research Training Network ‘Vacuolar Transport Equipment for Growth Regulation in Plants’ (MRTN-CT-2006–035833).

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Oral Communication Abstract – 2B.06

ARABIDOPSIS THALIANA GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AS AN OXIDATIVE STRESS SENSOR

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GAPC, cadmium, oxidative stress, Arabidopsis thaliana

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well known enzyme mainly involved in the glycolytic process. In mammalian cells, the GAPDH has been demonstrated also to play a role in the induction of apoptotic events. In particular, stimuli inducing oxidative stress have shown to induce nitrosylation of the GAPDH catalytic cysteine, leading to the enzyme inactivation and its relocalization into the nucleus, where it participates in the induction of apoptotic processes. In plants, both cytoplasmic and chloroplast GAPDH isoforms have been described, but up to now there are no evidences of their involvement in the induction of plant cell death events, even if nitrosylation of the same cysteine has been reported.

Cd^{2+} is a common environmental pollutant able to induce oxidative stress in plant cells with production of both reactive oxygen species and nitric oxide, leading to the induction of a senescence-like programme in cell cultures.

In order to investigate the possible involvement of plant GAPDHs in the Cd^{2+}-induced oxidative stress sensing, we focused on the *Arabidopsis* GAPC-1, one the two cytosolic GAPDH isoforms.

By performing *in vitro* analyses, using recombinant GAPC-1, we observed a reversible enzyme inactivation mediated by H_2O_2 and NO administration. The exposure of *Arabidopsis* seedlings to Cd^{2+} led to an accumulation of H_2O_2 and NO in roots where also an enhanced *GAPC-1* transcription and GAPC-1-YFP chimeric protein was detected, followed by its nuclear relocalization. In the *gapc-2* null mutant, where only the GAPC-1 enzyme is present, the Cd^{2+} stress determined an increase of GAPDH activity. Scavenging of H_2O_2 and NO in Cd^{2+} treated seedlings prevented the GAPC-1 accumulation.

Together these results support the hypothesis that the regulation of expression and activity of GAPC-1, in response to Cd^{2+}-induced oxidative stress, is mediated by the levels of H_2O_2 and NO in the cell that are directly sensed by the GAPC-1 enzyme.

We therefore propose that the GAPC-1 can be considered as an oxidative stress sensor.

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Poster Communication Abstract – 2B.07

MECHANISM OF GAMMA-ZEIN PROTEIN BODY FORMATION WITHIN THE ENDOPLASMIC RETICULUM

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Endoplasmic reticulum, protein bodies, transcriptomics, unfolded protein response, zein

Protein bodies (PB) are formed within the endoplasmic reticulum (ER) lumen by the extensive polymerization and insolubilization of seed storage proteins of the prolamin class, a process occurring in the seed endosperm cells of a number of cereals. PB-forming proteins have been exploited for the high accumulation of recombinant proteins, but the detailed mechanism of PB formation and ER retention is not yet fully clarified. Although PBs are formed by the assembly of many different polypeptides, certain individual prolamins, such as maize gamma-zein, are able to form PB also when expressed ectopically. We show that polymerization and insolubilization of gamma-zein requires cystein residues that are located in the N-terminal domain and are involved in inter-chain disulfide bonds. We have previously shown that this domain can promote PB formation when fused to the otherwise soluble vacuolar storage protein phaseolin, in the chimeric fusion protein zeolin. In an effort to identify ER proteins involved as helpers of PB formation, we have now studied the changes in the Arabidopsis transcriptome upon zeolin expression. Results will be presented in relation to the unfolded protein response that modulates ER activities in stressed situations.

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Poster Communication Abstract – 2B.08

PHASEOLIN: A MODEL PROTEIN FOR INVESTIGATION ON RECOMBINANT PROTEIN STABILITY IN THE CHLOROPLAST

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Chloroplast, folding, Nicotiana tabacum, phaseolin, protein stability

Plastid genetic engineering is a research area of plant biotechnology where, since the development of a chloroplast transformation protocol for tobacco, significant advances have been accomplished. Plastids offer considerable advantages as compared to conventional transgenic technologies, including high protein expression levels. Moreover, plastid transformation has been used to study the mechanisms of plastid gene expression and the plastid gene functions. However, the knowledge of the mechanisms regulating foreign protein folding, targeting, and accumulation in plastids is still quite limited. When a heterologous gene is inserted into the plastome and the corresponding protein is synthesized, the correct folding of this protein is very uncertain in the new environment. The stability of the foreign protein within the plastid environment seems to be the major determinant for accumulation. To investigate which are the most important molecular mechanisms that influence the stability of heterologous proteins in transplastomic plants, phaseolin has been used as reporter protein. Four gene constructs have been assembled and used to transform the tobacco plastome. One expresses the wild-type form of phaseolin with its endoplasmic reticulum (ER) signal peptide (spPhaseolin), which should target it to the thylakoids, as observed for zeolin (De Marchis et al., Plant Mol. Biol., 2010). The second gene construct is a mutated form of phaseolin which is able to form disulphide bonds thanks to a cysteine residue inserted at the C terminus (spPhaseolin*). We have preliminarily verified the formation of disulphide bonds between spPhaseolin* monomers in the ER. Moreover, other two phaseolin mutants have been prepared whose localization should be in the stroma because they will be devoid of their sp (ΔPhaseolin and ΔPhaseolin*). Based on previous results, the mutant ΔPhaseolin should be the most unstable form of phaseolin. The transplastomic plants will be analysed to investigate the stability of the phaseolin proteins, their folding and post-translational modifications.

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Poster Communication Abstract – 2B.09

TWO DIMENSIONAL LIQUID CHROMATOGRAPHY TECHNIQUE COUPLED WITH MASS SPECTROMETRY ANALYSIS TO COMPARE THE PROTEOMIC RESPONSE TO CADMIUM STRESS IN POPLAR

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Plant proteomics, two-dimensional liquid chromatography (2D-LC), cadmium treatment, poplar, phytoremediation

Plants are useful in studies of metal toxicity, because their physiological responses to different metals are correlated with the metal exposure dose and chemical state. Moreover a network of proteins and biochemical cascades that may lead to a controlled homeostasis of metals has been identified in many plant species. This paper focuses on the global protein variations that occur in a *Populus nigra* spp. clone (Poli) that has an exceptional tolerance to the presence of cadmium.

Using comparative proteomics, protein variations of the *Populus nigra* clone “Poli” grown without Cd, were compared to clones grown with a 50 μM $CdSO_4$ treatment, which was sub-lethal treatment for this clone. Protein separation was based on a two dimensional liquid chromatography technique (2D-LC) (2). The protein patterns obtained from plant samples of the two experimental conditions were compared using the DeltaVue software. The combination of a software that could quantify protein differences after 2D-LC and identify proteins, with MS technique gave both qualitative and semi-quantitative evidence of some of the proteome changes due to Cd treatment in this particular poplar clone. Three sets of proteins were characterized: *i)* more abundant in the treated sample in respect to the control, *ii)* less abundant in the treated sample in respect to the control, *iii)* equally abundant in both treated and control samples. A subset of 20 out of 126 peaks regulated differently under cadmium stress were digested with trypsin and identified by Matrix-assisted LASER desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS). Proteins that were more abundant in the treated samples were located in the chloroplast and in the mitochondrion, suggesting the importance of these organelles in the response and adaptation to metal stress.

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Poster Communication Abstract – 2B.10

BIOGENESIS OF THE ATKCO3 POTASSIUM CHANNEL

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Potassium channel, vacuole biogenesis, ABC transporter, protein turnover

Plant cell homeostasis is maintained by the activity of channels and transporters. These proteins must be specifically targeted, sorted and retained at appropriate membrane domains to control the vectorial transport of fluid, solutes, and electrolytes. The extent of permanence at the site of action could also be regulated, through interactions with the cytoskeleton or other associated proteins. Therefore, targeting signals as well as signals that control turnover coexist on the polypeptide. We are studying the Arabidopsis AtKCO3 potassium channel as a model to identify targeting and turnover signals as well as possible interactors. AtKCO3 is a single pore channel with two transmembrane domains and the N- and C-terminal regions exposed in the cytosol. A 14-3-3 binding region and two EF-hands are predicted at the N- and C-terminal domains, respectively. An AtKCO3::GFP fusion was previously found to be located at the tonoplast by transient expression. By subcellular fractionation, we confirmed the tonoplast localization of overexpressed AtKCO3 and the AtKCO3::GFP fusion in Arabidopsis transgenic plants. We determined that both AtKCO3 and AtKCO3::GFP form dimers in transgenic plants and in transiently transfected protoplasts (from Arabidopsis colture or tobacco leaves). Because four pores are necessary in a functional channel, the results indicate that most probably KCO3 is not functional by itself. Our electrophysiological studies confirmed that KCO3 and KCO3::GFP are silent channels. To identify potential partners involved in the regulation of (or regulated by) KCO3 we are performing yeast two hybrid screening using the last 87 AA of KCO3 as bait. We also identified a putative PDZ-binding motif of class 1 (-X-S/T-X-F) at the C-terminus of AtKCO3. PDZ proteins act as adaptors that facilitate signaling or determine the localization of receptors, channels, transporters and other signalling molecules. We are determining the turnover and half-life of AtKCO3 and a mutated form deleted of the putative PDZ-binding domain.

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Poster Communication Abstract – 2B.11

NUCLEAR TRANSFORMATION OF TWO CELL WALL-LESS STRAINS OF *CHLAMYDOMONAS REINHARDTII* FOR THE OVEREXPRESSION OF TWO PHYTOENE SYNTHASE EXOGENOUS GENES

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Nuclear transformation, Chlamydomonas reinhardtii, phytoene synthase, carotenoids

Microalgae have remarkable metabolic plasticity, easy and rapid growth capacity, which have induced the researches to focus on possible bioaccumulation abilities of highly productive strains, also considering the opportunities offered by genetic transformation. In microalgae, the chloroplast can represent an interesting compartment for the accumulation of molecules of interest, such as carotenoids ("cell factory"). The aim of this work was the genetic nuclear transformation of the green microalga *Chlamydomonas reinhardtii* cw-less strains in order to obtain carotenoid accumulation inside the chloroplast, through the expression of a key-enzyme involved in the carotenoid biosynthetic pathway, the phytoene synthase (PSY).

Two different expression vectors were obtained via Gateway Technology recombination and used for the nuclear transformation of cc-3491 and cc-400 cw-less strains, using glass beads method:

- pPSYB: β-tubulin promoter + intron *rbcs2* + cDNA enconing for *AtPSY* + *rbcs2* terminator + *aph7''*
- pPSYD: β-tubulin promoter + intron *rbcs2* + cDNA enconing for *OsPSY1* + *rbcs2* terminator + *aph7''*

After the transformation, Hygromycin-resistant colonies appeared for both strains, but a higher and more stable transformation efficiency was obtained for cc-3491, with a percentage of Hygro-PSY positive colonies of 60% for pPSYB and 100% for pPSYD Hygro-resistant colonies.

At present, a yellowish phenotype has been observed in only one transformant, B3, deriving from cc-3491 transformed with pPSYB, grown in liquid medium for two months. The photosynthetic pigment analysis revealed the same amount of chlorophylls, but a higher content of carotenoids (+40%) with respect to the control. New inocula from this culture were monitored at 5, 14 and 30 days after the inocula, showing at the 30th day a higher level of pigments with respect to the control (+62% chlorophylls, +25% carotenoids). Moreover, the PAM fluorimetry of B3 revealed a lower proportion of photoinactivated PSII after 5 min of high light exposure (1400 μmol m^{-2} s^{-1}), indicating a more effective photoprotection to high light. This is probably due to a different quantity and/or quality of carotenoids. Biochemical analyses are planned in order to confirm the presence of

the exogenous PSY protein in B3, and HPLC analysis will be performed in order to determine the composition of carotenoids.