Research Doctorate School in Biological and molecular Sciences

Student: Dr.ssa Valentina LUCAROTTI

Supervisors: Prof. Marcel KUNTZ
Prof. Pierdomenico PERATA

Doctorate Course: Molecular Biotechnology

Proteomic analysis of cold stressed
Arabidopsis thaliana chloroplasts

Laboratory: Dipartimento di biologia delle piante agrarie

Tutor: Prof. Amedeo ALPI

Abstract

Low temperature is one of the major abiotic stresses limiting the productivity and the geographical distribution of many species. The effect of cold acclimatization is evident at chloroplast level because one of the main target of damages causes by low temperatures is the photosynthetic apparatus. For this reason our aim is to analyze the change in level of expression of chloroplast proteins during stress. 13-days old plants were acclimatized at 4°C for 1 week and treated at -10°C for 12 h and then recovered for 24 h. Freezing treatment produced stress phenotypes of rolling leaves, decrease in pigment content. At low temperature wild-type plants exhibited symptoms of severe oxidative stress: lipid peroxidation, chlorophyll bleaching, and photoinhibition. In acclimatated plants, which accumulate over twice as much zeaxanthin as the control, these symptoms were significantly ameliorated.

The changes of total proteins in chloroplasts were examined using two-dimensional electrophoresis. Among 200 protein spots reproducibly detected on each gel, we found up- and down-regulated spots. Mass spectrometry analysis allowed the identification of 30 differentially expressed proteins, including well know cold-responsive proteins. Several proteins showed enhanced degradation during freezing stress, especially the photosynthetic proteins such as Rubisco activase (RcbA) and Rubisco large subunit (RcbL) of which 4 fragments were detected. The identified proteins are involved in several processes: photosynthesis, RNA processing, protein translation and processing, metabolism of carbon, nitrogen end energy. These proteins might work cooperatively to reach an homeostatic equilibrium to overcome stress conditions.

INDEX

Chapter 1   Introduction

Section 1- Plants and Cold stress

Section 2- Chloroplasts

Section 3- *Arabidopsis thaliana* as a model organism for plant proteome research

Chapter 2 Materials and Methods

Chapter 1 Introduction

Section 1- Plants and Cold stress

1.1 Plants responses at low temperatures

Low temperature severely affects plant growth and development, and limits crop distribution and yield. This adverse environmental condition is particularly important in temperate regions where significant temperature changes are frequent during the transition between warm and cold seasons (1).

Species adapted to cold environments by natural selection have evolved a number of physiological and morphological means to improve survival during extended cold periods. Typically, these species – herbs, grasses and ground shrubs – are of short stature, have a low leaf surface area and a high root⁄ shoot ratio. Their growth habit takes full advantage of any heat emitted from the ground during the day and minimises night chilling, since air temperature is maintained most effectively near the soil surface.

Low temperature leads to cellular dehydration, during ice formation (2). The effect of ice inside plant cells is devastating. Freeze-tolerant plants have several strategies to reduce the probability of this occurring, even when air temperature drops below zero, including maintaining high intracellular solute concentrations and encouraging ice nucleation outside the cells (2). These plants also commonly exhibit xerophytic adaptations to survive the reduced water availability within the plant and the soil. Temperatures of −5°C can kill an unhardened winter wheat plant even though it has the genetic capacity to acclimatize, harden and acquire tolerance of freezing down to −20°C. The cold-hardening mechanisms conferring freeze tolerance have been described and include changes in lipid composition, increases in

active-oxygen-scavenging enzymes (3), anthocyanin accumulation and altered growth morphology (4).

Many crops cultivated in temperate climates (e.g. maize, tomato, cucumber and mango) come from tropical and subtropical evolutionary backgrounds. These species apparently lack the genetic information to be or become freeze or even chill tolerant. These thermophilic crops thus offer the opportunity to study the effects of chilling on photosynthesis relatively undisguised by the gamut of protective and other acclimatory responses observed in chilling tolerant species (5).

Occasional short chilling episodes within a generally clement temperature environment are typical in many temperate regions where thermophilic crops are grown (e.g. maize in the Midwest USA). This is different to the suboptimum temperatures (e.g. <18°C) that persist for much of the growing season when warm-climate crops are planted at the low-temperature margins of their geographical range (e.g. maize in northern Europe). The underlying effects of these two chilling circumstances differ substantially.

There are reports that chilling can disrupt essentially all major components of photosynthesis including thylakoid electron transport, the carbon reduction cycle and control of stomatal conductance (6, 7). One of the important challenges to research in this field is identifying the primary effects within this highly interactive and regulated system that actually underlie the in vivo disorder. For example, stomatal closure following a chill could be a direct low temperature effect on guard cell function or an indirect response to a rising internal leaf CO_2 concentration (c_i) caused by a chill-induced loss of Rubisco activity (8).

Studying the effects of chilling in the dark on subsequent photosynthesis is important partly because plants in natural and agricultural habitats generally experience the lowest temperatures at night. At a specific low

temperature, the effects of concurrent light are typically greater and therefore are likely to mask those induced by chilling alone. Consequently, at a particular low temperature, there are substantial differences between plants chilled in the light and in the dark in both the scale of the inhibition of photosynthesis and the primary mechanisms involved (9).

Cold-adapted plants tend to be slow growing, have the C3 mode of photosynthesis and store sugars in underground tissues. Plants well adapted to cold environments have evolved an efficient respiration system, which allows them to rapidly mobilise stored reserves during the short growing season.

The timing of developmental and physiological responses to environmental stress is under strict genetic control. In fact, the process of cold acclimation is very complex and involves extensive physiological and biochemical changes (10;11). While some of these changes are implicated in the stability of subcellular structures, essentially membranes (12) and cytoskeleton, others are directed to control the synthesis of pigments to protect from photoxidative stress (13-17), enzymes to reduce oxidative stress (18, 19), and compatible solutes to protect from osmotic changes. In addition, during cold acclimation there is an increase in the levels of cryoprotective proteins, and antifreezing proteins. Most of these alterations are regulated by low temperature through changes in gene expression. In this regard, whole transcriptome analyses in *Arabidopsis* have shown that around 1000 genes are regulated by low temperature (20). Many early cold-regulated genes encode transcription factors or proteins involved in transcription (21), which suggests that the process of cold acclimation is subjected to a complex transcriptional regulation and involves multiple regulatory pathways (22). Furthermore, it is worthy to mention that an important number of genes that are regulated by low temperature are also regulated by other abiotic stresses

such as dehydration and high salt, indicating that plant responses to different abiotic stresses are related and share common signalling pathways (Fig.1).
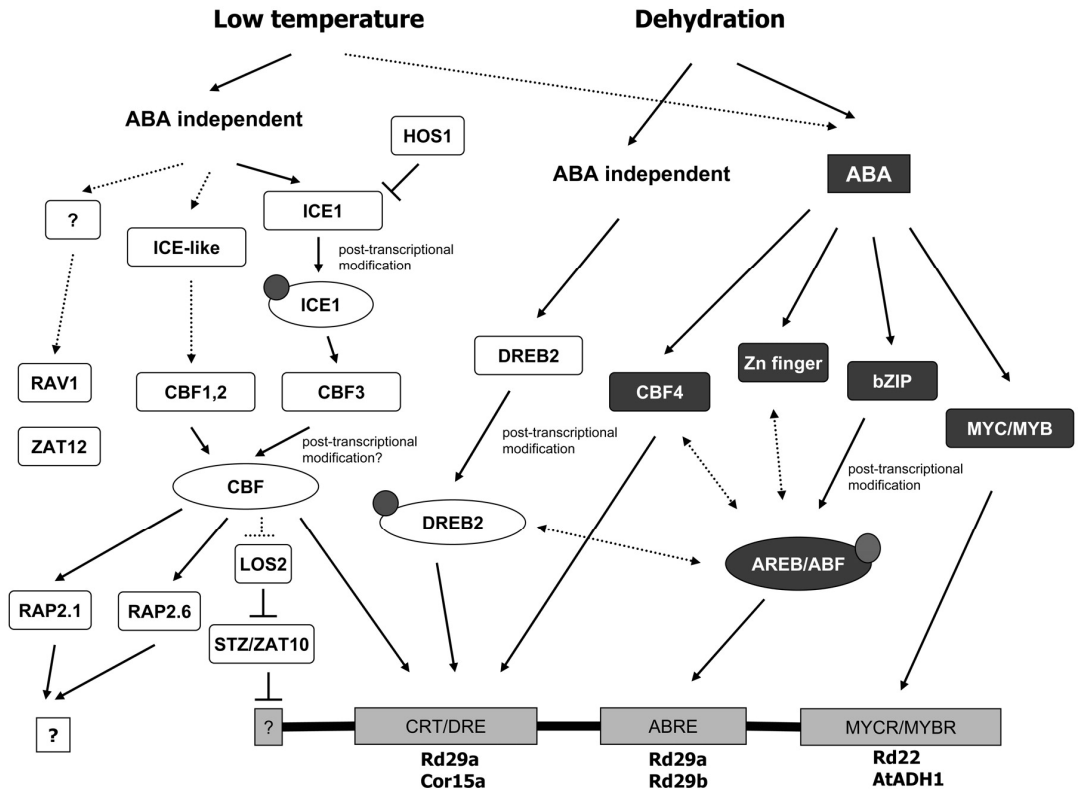
Figure 1. Transcriptional cascades of low temperature and dehydration signal transduction. ABA-dependent transcriptional factors are shaded, while ABA independent factors are not. Small circles indicate posttranscriptional modification, such as phosphorylation. Transcription factor binding sites are represented as rectangles at the bottom of the figure, with the representative promoters listed below. Dotted lines indicate possible regulation. Double arrow lines indicate possible cross talk (Zhang *et al.,* 2004 (23)).

Understanding the molecular mechanisms controlling cold acclimation, therefore, should provide essential information on how plants co-ordinately

integrate responses to different unfavourable environmental conditions to develop and reproduce correctly, and has the biotechnological potential to generate molecular tools to improve crop tolerance to abiotic stresses.

A major step toward the understanding of how gene expression is regulated during cold acclimation was the identification in *Arabidopsis* of a small family of three transcriptional activators known as C-repeat-binding factors (CBFs)/dehydration-responsive element-binding factors 1 (DREB1s) (24–25). These factors bind to the low temperature responsive DNA regulatory element termed C-repeat (CRT)/dehydration response element (DRE) (26), which is present in the promoters of many cold-inducible genes (27). Constitutive over expression of any CBF gene in *Arabidopsis* induces the accumulation of mRNAs from genes that contain the CRT/DRE motif in their promoters, the CBF regulon, as well as an increase in freezing tolerance (28). The CBFs have been described to regulate the expression of around 12% of the *Arabidopsis* cold-inducible genes (27), which gives an idea about their relevance in cold acclimation.

1.2 Low temperature and photosynthesis

Photosynthesis is the principal mechanism to transform light energy into biochemical usable chemical potential energy (ATP) and redox potential energy (NADPH) by components of the chloroplast thylakoid membrane (Fig. 2). NADPH and ATP are then transferred to consumed in metabolic electron sinks. The main metabolic sink occurs via reduction of CO_2 through the Calvin cycle to form carbohydrates within the stroma of the chloroplast. The primary reactions of photosynthesis are temperature independent and catalysed by photosystem (PS) I and II to trap light energy and transform it into redox potential energy through a combination of extremely rapid (10^{-15} -10^{-12} s) photophysical and photochemical processes leading to charge

separation. In contrast, much slower (10^{-3} − $10°s$), temperature-dependent, biochemical reactions convert this redox potential energy to stable reducing power in the form of NADPH. Thereby a *trans*-thylakoid ΔpH is established by the oxygen-evolving complex and the plastoquinone (PQ) pool of the intersystem electron transport, to synthesize ATP by chemiosmosis. ATP and NADPH are then consumed in the reduction of CO_2 to triose phosphate and the continuous regeneration of ribulose-1,5-bisphosphate (RuBP) on a time scale of seconds. The energy represented by the fixed carbon is then used through maintenance respiration to maintain cellular homeostasis and growth respiration for cell division and expansion on a time scale of minutes to hours. Although C reduction is considered photosynthetic, it is also important to appreciate that N and S assimilation are also photosynthetic because of their dependence on photosynthetically generated redox and chemical potential energy (29).
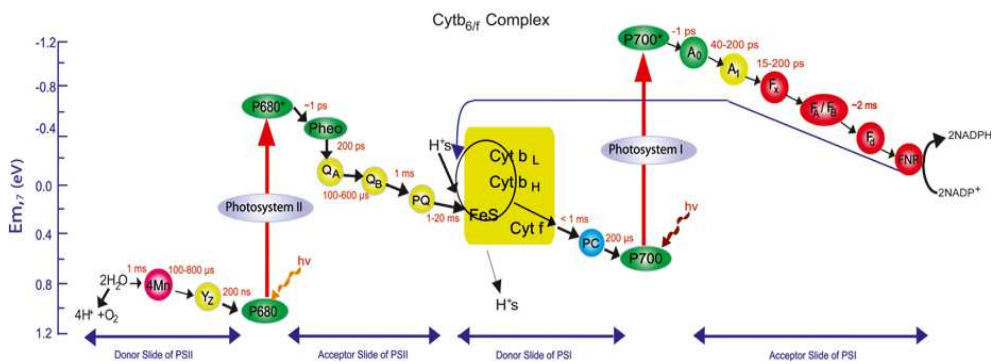
Figure 2. The Z-scheme of oxygenic photosynthesis for electron transfer from water to oxidized nicotinamide adenine dinucleotidephosphate (NADP). The symbols are: Mn = Mn cluster; Y = Tyrosine –161 on D1 protein; P680 = a pair of Chls the reaction center (RC) Chls of PS II having one of its absorption bands at 680 nm, P680* = excited P680; Pheo = the primary electron acceptor of PS II; QA = the primary plastoquinone electron acceptor of PS II; QB = secondary plastoquinone electron acceptor of PS II; PQ = plastoquinone pool; FeS = Rieske iron sulfur protein; Cyt f = cytochrome f; CytbII = high potential cytochrome b6; PC = plastocyanin; P700 = a pair of Chl a and Chl a¢ the RC Chls of PS I; P700* = excited P700; A0 =

primary electron acceptor of PS I a Chl monomer; A1 = secondary electron acceptor of PS I vitamin K; Fx FA and FB = three different iron sulfur centers; Fd = ferredoxin; and FNR = ferredoxin NADP reductase. Approximate estimated times = various steps are also noted on the figure. A circular path in the Cyt b6f complex symbolizes the existence of a Q-cycle; and a dotted line from the electron acceptor side of PS I to the PQ/ Cyt b6f region symbolizes the existence of a cyclic flow around PS I under certain conditions. Reproduced from Govindjee (2004) (30).

Optimum plant performance requires a balance in the rates of source versus sink processes. Low temperatures, however, can inhibit electron transport by increasing membrane viscosity through alterations in the biophysical properties of thylakoid lipids and decrease the rates of the enzymatic reactions involved in C, N and S reduction more strongly than they inhibit photophysical and photochemical processes involved in light absorption, energy transfer and transformation (31). On the whole plant level, low temperatures affect the rate of growth through an inhibition of the uptake of water and nutrients. As a consequence of low temperatures, the rates of photochemical processes need to adjust to the decreased metabolic sink capacity for the consumption of photosynthates. Thus, the effects of low temperatures on photosynthesis and carbon gain eventually determine productivity, growth and distribution of plants on various spatial scales (32). Cold tolerant species are able to acclimate to low temperatures. Mechanisms to overcome the constraints of short-term and long-term exposure to low temperature include changes in energy absorption and photochemical transformation through energy partitioning and concomitant changes in chloroplastic carbon metabolism, allocation and partitioning. Thus, the mechanisms involved in photosynthetic acclimation to low temperature range from modifications within the thylakoid membrane system, affecting photosynthetic electron transport (33) to post-transcriptional activation and

increased expression of enzymes for sucrose synthesis, changed expression of Calvin cycle enzymes, changes in leaf protein content, as well as the signals that trigger these processes (34).

Photosynthesis reflects not only short-term responses of the chloroplast to the prevailing environmental conditions but also the physiological and developmental state of the whole organism. Cellular redox poise is one element in the puzzle which is linked, in the short term at least, to the accumulation of sugars at low temperatures. However, long-term acclimation of photosynthesis to low temperature in many plants also depends on development under low temperature. Plants adjust photosynthesis to low temperatures using key cellular features, source activity as reflected by the redox state of the chloroplast and sink strength as reflected in metabolic activity. In addition to the roles of source activity and sink strength in energy conversion, both also act as global sensors of environmental change and provide the signalling required to alter specific gene expression. Large-scale genetic analysis now reveals a complex, new picture of signalling pathways involved in the process of low temperature acclimation of photosynthesis and the network that actually controls photosynthesis (Fig. 3).
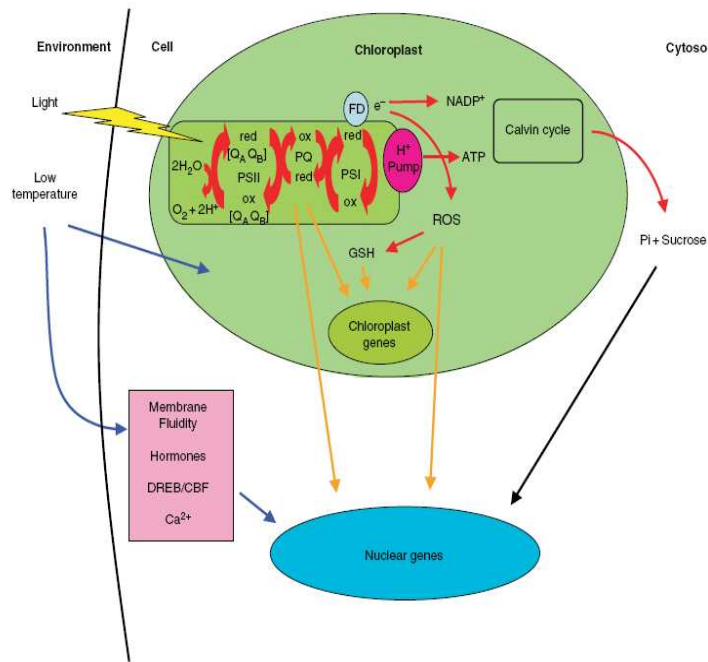
Figure 3. Integration and regulation of the photosynthetic process. The primary light reactions occur within the thylakoid membrane system, where the water splitting complex, PSII, PSI and the electron transport chain are located. Energy trapped by PSII is used to transfer electrons derived from the water-splitting process via the electron-transport chain by the mobile electron carrier plastoquinone (PQ). After a second light reaction in PSI the electrons can be accepted by NADPH via ferredoxin (FD). Protons are pumped across the thylakoid membrane to generate ATP. The main metabolic sink for NADPH and ATP is the Calvin cycle. Its sink strength depends on the rate of export of triose-P to the cytosol and subsequent export to sink tissues. Under increased excitation pressure, e.g. because of low temperature and/or high light intensities, electrons from PSI can also be transported to oxygen, thereby generating reactive oxygen species (ROS). ROS can be detoxified via gluthathione, causing changes in the ratio of the oxidized to the reduced state of gluthathione (GSH). Red arrows indicate the flow of energy, blue arrows indicate low temperature induced signals, orange arrows indicate redox-mediated signals and black arrows indicate sugar signals. From Ensminger *et al.*, 2006 (35).

1.3 Cold sensing and signalling

The identity of the plant sensors of low temperature remains as yet unknown (36). Multiple primary sensors may be involved, with each perceiving a specific aspect of the stress, and each involved in a distinct branch of the cold signalling pathway (37). Potential sensors include Ca^{2+} influx channels, two-component histidine kinase and receptors associated with G-proteins. Certain cytoskeletal components (microtubules and actin filaments) participate in cold sensing by modulating the activity of Ca^{2+} channels following membrane rigidification (38). Because of its basic role in separating the internal from the external environment, the plasma membrane has been considered as a site for the perception of temperature change, with its rigidification representing an early response. The phosphorylation of proteins, together with the suppression of protein phosphatase activity, may also provide a means for the plant to sense low temperatures. Thus, a variety of signalling pathways is triggered, including secondary messengers, ROS, Ca^{2+}-dependent protein kinases (CDPKs), mitogen-activated protein kinase (MAPK) cascades and the activation of transcription factors (TFs), all of which promote the production of cold-responsive proteins. These products can be divided into two distinct groups: regulatory proteins controlling the transduction of the cold stress signal, and proteins functionally involved in the tolerance response. The latter include LEA (late embryogenesis abundant) proteins, antifreeze proteins, mRNA-binding proteins, chaperones, detoxification enzymes, proteinase inhibitors, transporters, lipid-transfer proteins and enzymes required for osmoprotectant biosynthesis (39). An outline of these processes is given in Fig. 4.
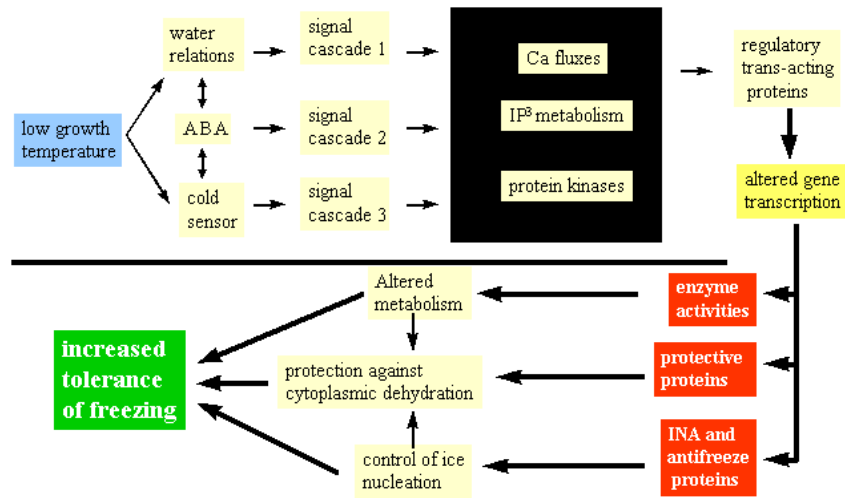
Figure 4. A model of the signalling processes in plants leading to acclimation and increased freezing tolerance. (From www.cepceb.ucr.edu)

1.4 Arabidopsis thaliana and cold stress

Interest in profiling the system response to low temperature has revealed thousands of transcript (40) and hundreds of metabolite (41) changes. These profiling technologies have also been used to characterize regulatory pathways. Global transcript profiling analyses indicate that >10% of genes in the *Arabidopsis* genome are regulated during cold acclimation (42). Experiments with Affymetrix ATH1 arrays led to 504 genes that are defined as a cold standard set of cold responsive genes (COS), 302 of which are up regulated and 212 down regulated, based on the criterion that up- (or down) regulation occurred in cold-treated plants grown on both soil and defined agar medium. The large number of genes identified raises the question of exactly which genes are most central to increasing freezing tolerance (43). Forward and reverse genetic approaches have defined some of the key regulatory components of cold acclimation. A set of genes that encode a family of cold-regulated (COR) proteins, are massively induced during cold

acclimation (44). These COR genes have also been named low-temperature-induced (LTI), cold acclimation-specific (CAS), cold-induced (Kin), and responsive to drought (RD) genes. Most of cold-responsive genes are regulated through C-repeat/dehydration-responsive elements (CRT/DRE) and abscisic acid-responsive element (ABRE) by a family of *Arabidopsis* transcription factors known as either C-repeat-binding factors (CBF1, CBF2 and CBF3) or dehydration responsive element-binding factors and by basic leucine zippers (bZIPs) (SGBF1). The ICE1–CBF transcriptional cascade has an important role in cold acclimation in diverse plant species (Fig. 5, from Chinnusamy *et al.,* 2007 (45)). ICE1 (inducer of CBF expression 1) encodes a MYC-like bHLH transcriptional activator that, in addition to the direct induction of CBF expression, also appears to regulate negatively MYB15, an upstream negative regulator of CBFs. ICE1 protein level and activity are regulated post-translationally by HOS1-mediated ubiquitination and proteolysis. Several CBF-independent regulons that are critical for cold acclimation have also been identified. Metabolism, and RNA processing and export, affect cold tolerance via cold signaling and/or cold-responsive gene expression (Fig. 4). The constitutive HOS9 and HOS10 regulons have a role in the negative regulation of CBF-target genes.
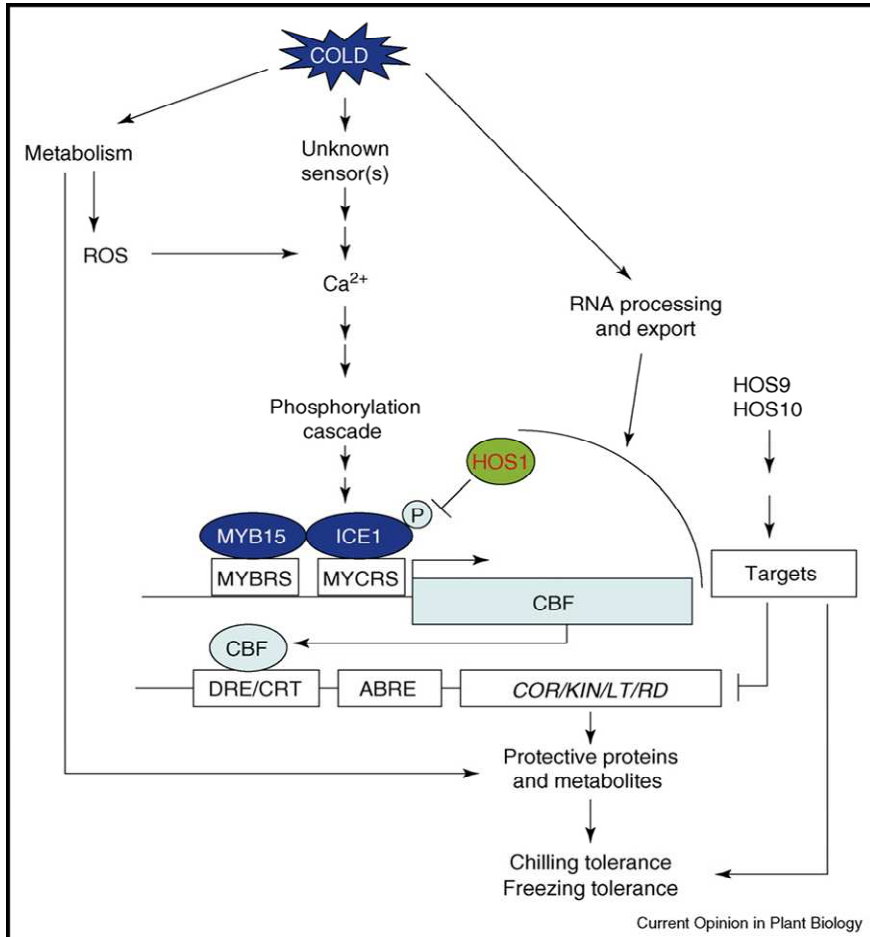
Figure 5. Schematic illustration of the cold response network in *Arabidopsis*.

Abbreviations: CBF, C-repeat binding factor (an AP2-type transcription factor); CRT, C-repeat elements; DRE, dehydration-responsive elements; HOS1, high expression of osmotically responsive genes1 (a RING finger ubiquitin E3 ligase); ICE1, inducer of CBF expression 1 (a MYC-type bHLH transcription factor); MYBRS, MYB transcription factor recognition sequence; P, phosphorylation; MYCRS, MYC recognition sequence (Chinnusamy *et al.,* 2007).

CBFs/DREBs are themselves transiently induced by low temperature (46). Therefore, there is a transcriptional cascade that leads to the expression of the RD/COR/KIN/LTI/CAS genes under cold stress (Fig. 6, from Xin *et al.,* 2007 (43)).
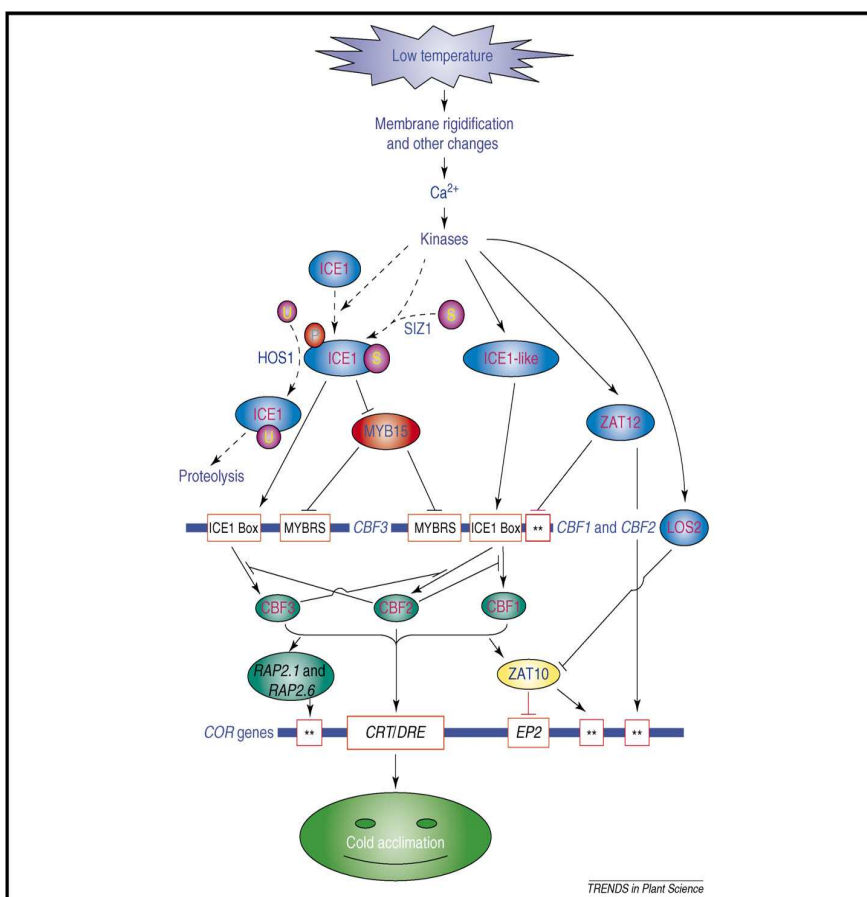
Figure 6. Diagram of cold-responsive transcriptional network in *Arabidopsis*. Plants probably sense low temperatures through membrane rigidification and/or other cellular changes, which might induce a calcium signature and activate protein kinases necessary for cold acclimation. Constitutively expressed ICE1 is activated by cold stress through sumoylation and phosphorylation. Cold stress induces sumoylation of ICE1 at K393, which is critical for ICE1-activation of transcription of CBFs and repression of MYB15. CBFs regulate the expression of COR genes that confer freezing tolerance. The expression of CBFs is negatively regulated by MYB15 and ZAT12. HOS1 mediates the ubiquitination and proteosomal degradation of ICE1 and, thus, negatively regulates CBF regulons. CBFs might cross-regulate the each other's transcription. CBFs induce the expression of ZAT10 (=STZ), which might downregulate the expression of COR genes. Cold-upregulated LOS2

represses the transcription of ZAT10. ZAT10 and ZAT12 are two C2H2 zinc finger transcription factors.

Broken arrows indicate post-translational regulation; solid arrows indicate activation, whereas lines ending with a bar show negative regulation; the two stars (**) indicate unknown cis-elements. Abbreviations: CBF, C-repeat binding factor (an AP2-type transcription factor); CRT, C-repeat elements; DRE, dehydration-responsive elements; HOS1, high expression of osmotically responsive genes1 (a RING finger ubiquitin E3 ligase); ICE1, inducer of CBF expression 1 (a MYC-type bHLH transcription factor); LOS2, low expression of osmotically responsive genes 2 (a bifunctional enolase with transcriptional repression activity); MYB, myeloblastosis; MYBRS, MYB transcription factor recognition sequence; SIZ1, SAP and MiZ1 (a SUMO E3 ligase); P, phosphorylation; S, SUMO (small ubiquitin-related modifier); U, ubiquitin. (Xin *et al.,* 2007)

CBF-dependent roles in freezing tolerance have also been demonstrated for LOS1, a translational elongation factor 2 gene, which is involved in protein synthesis in the cold (47), and for LOS4, a DEAD box RNA helicase that is essential for mRNA export (48). By a reverse genetic approaches was identified a gene defined by sfr2 (sensitive to freezing 2) mutation, which was reported to encode a β-glucosidase isozyme (49). The SFR2 gene is constitutively expressed in most aerial tissues and does not change in response to cold acclimation or other stress treatments. By reverse genetic approaches was also identified ESK1 (Eskimo 1), a member of a large family of plant specific genes whose function is unknown (43). ESK1 encodes a protein of 487 amino acids and predicted molecular mass of 56.7 kDa, working as negative regulator of cold acclimation. Mutations in this gene lead to freezing tolerance through mechanisms that are largely independent of those mediated by CBF2 overexpression (44) or expression of the COS gene set. Bioinformatic analysis indicates that the ESK1 protein has a

conserved domain of unknown function (DUF) 231 (Pfam PF03005) from amino acid 310 to 483, which is only found in proteins from higher plants.

In addition to transcriptional regulation, gene expression is regulated post-transcriptionally at pre-mRNA processing, mRNA stability, export from nucleus and translation steps (50). Regulation of gene expression at the post-transcriptional level is mainly achieved either directly, by RNA-binding proteins (RBPs), or indirectly, whereby RBPs modulate the function of other regulatory factors (45). Several conserved RNA-binding motifs have been discovered, such as the RNA recognition motif (RRM), glycine-rich motif, arginine-rich motif, RGG box, zinc finger motif, and double-stranded RNA-binding motif (51). RRMs occupy the N-terminal half of RBPs, and are involved in the recognition and binding of target RNA molecules. The C-terminal half of RBPs contains other auxiliary domains that may be involved in interaction with other protein factors. Proteins that contain RRMs in the N-terminal half and a glycine-rich region in the C-terminal half (glycine-rich RNA binding proteins; GRPs) have been described in plants including maize, *Arabidopsis thaliana*, tobacco, barley, Brasicca, leafy spurge and alfalfa. The *Arabidopsis* genome encodes 196 RRM-containing proteins, of which 27 are classified as glycine-rich and small RRM-containing proteins (52). Recently, Kim *et al.* (2007) have shown that an *Arabidopsis* GRP, designated atRZ-1a and GRP2 (accession number At4g13850), affect seed germination and seedling growth at low temperatures, and plays a role in the enhancement of freezing tolerance of *Arabidopsis* plants, demonstrating a role of GRP in plant responses to environmental stress. Proteome analysis revealed that expression of several mitochondrial-encoded genes was modulated by GRP2 indicating also that GRP2 exerts its function by modulating the expression and activity of various classes of genes. GRP2 contains an N-terminal RRMs and a C-terminal glycine-rich region.

However, it has different structural features compared with GRP4 and atRZ-1a, in that GRP2 has much longer glycine-rich domain than GRP4 and does not have the CCHC-type zinc fingers found in atRZ-1a.

Section 2- Chloroplasts

1.5 Chloroplasts

Plastids are semiautonomous organelles that are ubiquitously found in plant cells. They are derived from an endosymbiotic event and are thought to have evolved from an ancient photosynthetic prokaryote related to present-day cyanobacteria. Following endosymbiosis, the plastid genome has been reduced to 100 genes, mainly coding for housekeeping functions (translation and transcription of the plastid genome) and proteins required for primary photosynthetic reactions.

The most conspicuous plastid type is the chloroplast, found in leaves and carrying out photosynthesis as its main function is also responsible for various essential functions, including lipid metabolism, starch and amino acid biosynthesis. Photosynthesis is an integrated biological process involving the coordinated functioning of chloroplast compartments: **(a)** the thylakoids, a highly organized internal membrane network formed of flat compressed and connected vesicles where solar energy is collected and converted into stored chemical energy (ATP and NADPH) while oxygen, a by-product of the reactions, is evolved; **(b)** the stroma, an amorphous background rich in soluble proteins that is the site for the reduction of carbon dioxide and its conversion into carbohydrates; and **(c)** the envelope, a pair of membranes surrounding the chloroplast, that tightly controls the metabolic dialogue between the organelle and the rest of the cell (Fig. 7). Among chloroplast subfractions, the envelope membranes are rather unique

as they represent a minor chloroplast component (1–2% of the chloroplast proteins) playing a key role in chloroplast metabolism and biogenesis. However, the details of chloroplast functions and the compartmentation of chloroplast proteins are not yet fully understood, and there is a major interest in analyzing them for understanding regulation of whole plant cell metabolism. Furthermore, more proteins, new pathways, and their precise localization remain to be discovered. The green colour of plants is due to the green colour of chlorophyll, all of which is localized to the thylakoid membrane. A granum is a stack of adjacent thylakoids. The stroma is the space between the inner envelope membrane and the thylakoids.
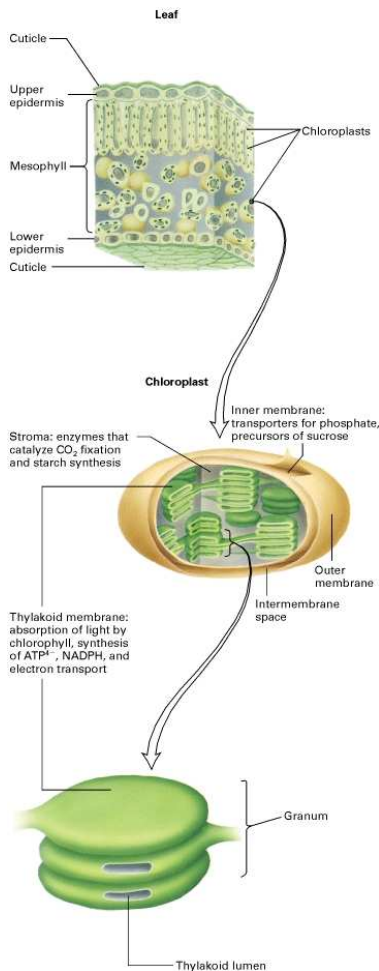

Figure 7. The structure of a leaf and chloroplast.

1.6 Chloroplasts isolation

Only structurally intact chloroplasts with a functional envelope exhibit metabolic activities comparable to those of the original tissue. Therefore, the most important objectives that should guide the isolation of chloroplasts from any plant species are their morphological and physiological integrity. The use of intact chloroplasts is absolutely essential for studying processes like light-driven CO_2 fixation, CO_2-dependent O_2 evolution, *in organello* RNA and protein synthesis, import of cytoplasmically made polypeptides into chloroplasts, or incorporation of labeled precursors to dissect the enzymatic machinery involved in fatty acid biosynthesis and fatty acid incorporation into chloroplast lipids.

In some plant species, like spinach or pea, highly active chloroplasts can be obtained by following several simple rules:

Use of young, fresh plant material free from starch

- Distruption of cells in a buffered medium containing sorbitol
- Rapid separation of chloroplasts from the remaining cell constituents

Unfortunately, the standard protocols for chloroplast isolation typically result in poor chloroplast yields, or chloroplasts of low activity, when applied to *Arabidopsis*. The reason for this apparent sensitivity of *Arabidopsis* chloroplasts are not known. However, concerted efforts of several research groups to optimize established chloroplast isolation protocols for *Arabidopsis* have been done. Nowadays we have two reliable procedures: chloroplast isolation by homogenization of leaf tissue and chloroplast isolation from protoplasts.

The development of methods for the isolation of functional chloroplasts from plants tissue has been a long process, starting from the late 1930s when

Robert Hill prepared chloroplast fractions shown to evolve oxygen in presence of an artificial oxidant, but not in presence of CO_2 (53, 54). For a long time, chloroplast preparation was restricted to the isolation of green particles capable of supporting the Hill reaction. Arnon and coworkers (55) demonstrated CO_2-dependent O_2 evolution by chloroplast suspension, but the rates were considerably lower than those of intact leaves. It is only from when the importance of maintaining chloroplast integrity throughout the preparation process was fully recognized that preparations showing high rates of CO_2-dependent O_2 evolution were obtained (56). Indeed, the use of sugar as an osmoticum (as suggested initially by Hill) together with a very brief homogenization and rapid separation allowed the isolation of chloroplasts with an intact envelope (57). Furthermore, Jensen and Bassham (1966, (58)) significantly improved the medium, especially the buffer, used for chloroplast isolation. Thus, in the late 1960s, methods to prepare physiologically active chloroplasts by differential centrifugation were widely available. Indeed, such chloroplast preparations paved the way for an extensive characterization of metabolite transport and mechanisms across the envelope membranes, studies of protein, and lipid synthesis. For instance, Heber and Santarius (1970, (59)) demonstrated the exchange of ATP and ADP across the chloroplast envelope, whereas Heldt and Sauer (1971, (60)) identified the inner chloroplast envelope membrane as the site of specific metabolite transport. We should also mention the parallel development of non-aqueous methods to prepare chloroplast, with the aim of analyzing the intracellular distribution of water-soluble molecules between plastid and cytosol. Despite their interest, the development of such procedures remained limited.

Although physiologically and structurally intact, chloroplasts fractions prepared by differential centrifugation were actually rather crude because

they contained pieces derived from other cell compartments (e.g., nuclei and mitochondria) and even a few intact cells. The next step was therefore to remove these contaminants and prepare chloroplasts as pure as possible. Sucrose was first used for preparing gradients to separate chloroplasts, and than various media like colloidal silica-derived compounds were developed. Unfortunately, chloroplasts purified on sucrose gradients were unable to perform CO_2-dependent oxygen evolution, mostly because the envelope became leaky during the course of the centrifugation at a high sucrose concentration. Actually, the development of Percoll (61) was the major breakthrough that allowed the development of procedures to prepare intact and almost pure chloroplasts from a wide variety of tissues. Percoll-purified chloroplasts were able to achieve CO_2-dependent oxygen evolution almost identical to that of the leaves (on a chlorophyll basis). Percoll has several key characteristics for the purification of physiologically active organelles:

1. Percoll is made of silica beads of various size and is highly suitable to the formation of density gradients that can be self generated upon high-speed centrifugation;

2. Percoll is an inert compound, its low osmolarity does not change that of the assay medium, even at a high concentration, thus maintaining the integrity of the organelle structure during density gradient centrifugation;

3. in Percoll, silica beads are coated with polyvinylpyrrolidone, which helps to remove harmful phenolic compounds from the medium.

In the early 1990s, a large number of publications were produced with the term "Percoll" as a key word. Screening the literature with the term "Percoll" now identifies >5000 published papers, thus demonstrating the importance of a powerful method – density gradient centrifugation – applicable to any cells or organelles in suspension for which differences in size or buoyant density exist.

Several protocols are described to perform Percoll purification of pure and intact chloroplast from spinach or pea leaves. More recently, and since the complete sequencing of the *Arabidopsis* genome (The AGI, 2000), *Arabidopsis thaliana* has become a widely used model organism, supplanting spinach and pea as model plants. Somerville *et al.* (1981, (62)) had already described the isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*. However, the method did not rely on the use of Percoll-gradients to isolate the organelles. More recently, Kunst (1998, (63)) and Ferro *et al.* (2003, (64)) published alternative methods for the Percoll-based purification of chloroplasts from *Arabidopsis*.

Critical parameters

There are several essential points to take into consideration for successful purification of chloroplasts, for instance, the leaf material, medium composition are critical steps of protocol.

The best results are obtained when starting from 3- to 4-week-old *Arabidopsis* rosette leaves. Leaves that are too old are enriched in phenolic compounds that are known to affect integrity of the chloroplasts. It however appears that starting from younger leaves improves yield, purity and integrity of the purified organelles.

The quantity of leaves is also critical. No intact chloroplasts can be recovered when the leaf starting material is <60 g.

The number of starch granules present in chloroplasts is a problem for the preparation of intact chloroplasts: in fact these plastids containing large starch grains will generally be broken during centrifugation. Therefore, prior to the experiment, the plants should be kept for several hours in a dark and cold room (4 °C) to reduce the amount of starch. A good way to proceed is to

place the plants under such condition the day before the extraction (usually at the beginning of the afternoon of the day before).

The tissue must remain cold throughout the protocol to help reduce endogenous protease activities.

The grinding process must be as short as possible. Longer blending improves the yield of recovered chlorophyll, but increases the proportion of broken chloroplasts. The crude chloroplast suspension obtained after homogenization of leaves that are too young, is mainly composed of broken chloroplasts. When leaf material has been homogenized, the homogenate should rapidly be filtered and centrifuged to protect chloroplasts from activities of proteases released after disruption of the cells. Addition of BSA immediately before use of the grinding medium helps to limit the activity of proteases.

The filtration process should also be rapidly performed. After the grinding process, organelles are released in a crude extract consisting of broken cells, having released some very aggressive compounds (e.g. protease, lipase and phenolic compounds). Storing the organelles for too long in such a homogenate strongly affects their integrity and functioning. Therefore, a short delay between grinding and concentration is expected to improve yield of intact chloroplasts (Fig. 8).
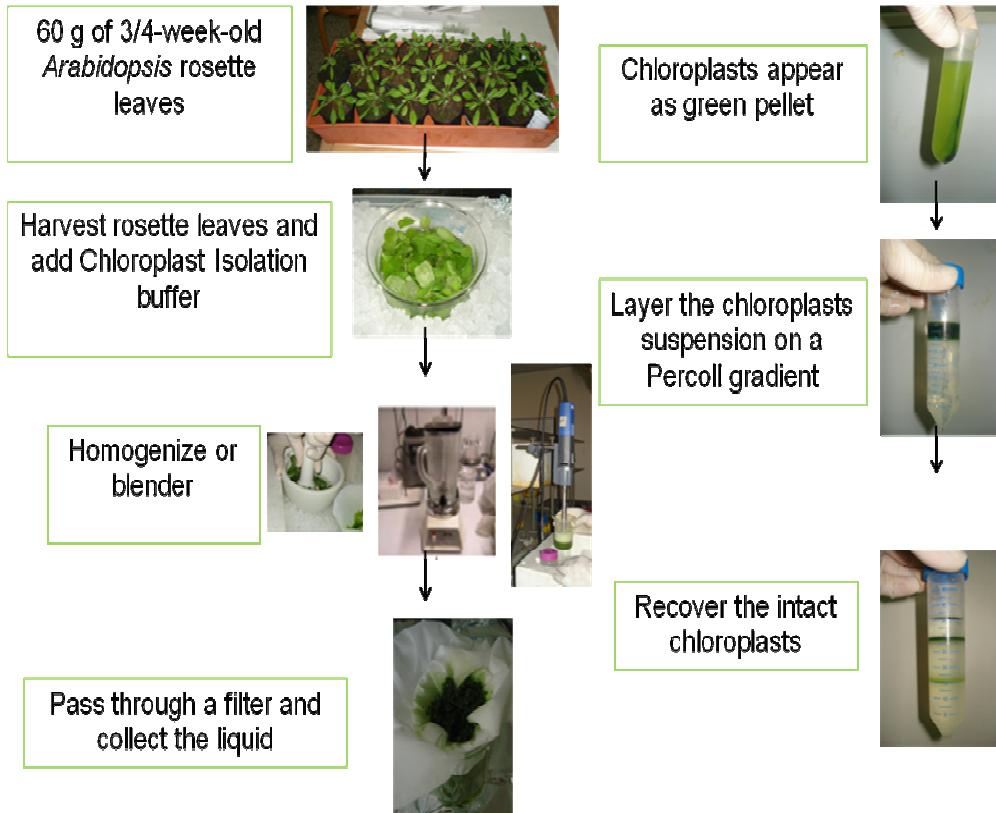
Figure 8. **Strategy used to purify intact chloroplasts from *Arabidopsis* leaves.**

Section 3- *Arabidopsis thaliana* as a model organism for plant proteome research

1.7 Proteomics

Proteomics defines an approach for the systematic identification of all proteins expressed in a cell and is expected to accelerate discoveries in the life sciences. The progress of proteomics and proteomics-related technologies over the last decade is based on two major developments. First, the wealth of genome information paved the way for the large scale analysis of proteins for which amino acid sequences were deposited into databases (*Arabidopsis* Genome Initiative, 2000). Second, technological improvements

in mass spectrometry, especially the development of soft ionization techniques for peptide analysis, allowed rapid and sensitive protein identification from minute quantities of biological samples (reviewed in Aebersold and Mann, 2003 (65)). Although it is generally accepted that the proteome is dynamic and difficult to define, scientists aim at the most complete identification of the protein complement of a cell or a tissue type under certain, well-defined conditions.

Proteomics provides an excellent opportunity to study the response of plants to environmental stress and to identify stress-responsive proteins.

Proteomics, the comprehensive and quantitative analysis of proteins that are expressed in a given organ, tissue, or cell line, provides unique insights into biological systems that cannot be provided by genomic or transcriptomic approaches. The fast developments in the 'omics' area and the combination of different 'omics' tools offer great potential for post-genomics to elucidate the genotype-phenotype relationships for both fundamental and applied research. Consequently, large sets of integrative data have been acquired, providing information about proteins and metabolites involved in developmental and environmental responses.

1.8 Proteomic strategies in the post-genomic era

Arabidopsis was the first established model plant worldwide. The comprehensive resources which are available for *Arabidopsis*, such as the entire genome sequence, a larger collection of natural variants and an ever increasing number of molecular tools, made it the favourable model for post-genomic research. Thus, several fundamental information platforms and databases already exist such as TAIR (http://www.*Arabidopsis*.org/).

The availability of a whole genome sequence in *Arabidopsis* provides unique opportunities for genome-based systems biology approaches. It enables the

use of post-genomic tools such as proteomics in its full capacity. The introduction of shotgun proteomics enables the high-throughput analysis of large sets of samples along with the identification of several hundreds of proteins within each sample. The latter approach, in conjunction with the constantly increasing availability of genomic sequences, has rapidly become a favourable tool for many researchers. An initiative to assist in the coordination of international proteomics research on *Arabidopsis thaliana* has been established, leading to the assembly of the largest proteomics resource for a plant model system to date (66). This major proteomics platform, provided by the Multinational *Arabidopsis* Steering Committee Proteomics, is available on line (MASCP; http://www.masc-proteomics.org/).

In the last few years one of the most active proteomic approaches within the *Arabidopsis* research field consisted of profiling isolated cell organelles. They stand for an important organizational level of the plant cell proteome. Proteome analyses so far encompassed virtually all organelles in plant cell including peroxisomes, vacuolar membranes, the plasma membrane, the cell wall, mitochondria, nucleous and nucleolus and different plastid types. Mitochondria and chloroplasts have dominated the field of sub-cellular proteomics and are inevitably the best characterized plant cell organelles at the proteome level. Reasons for this are the numerous dedicated studies that were designed to map the complete protein complement of these two organelles, which has led to the high proteome coverage available to date. Furthermore, excellent sub-organellar compartment characterization has been achieved by proteomic characterization. And finally, several quantitative approaches were taken to analyze the dynamic adaptation of the proteome to changes in environmental conditions. Thus, for these two organelles we have a vague idea how the plant adjusts and controls the

proteome of mitochondria and chloroplast or the whole plant for a selected set of environmental conditions.

Despite the impressive progress in the field of organelles there are several open questions that remain to be addressed and we would like to highlight some of them by taking a closer look at the current status of plastid proteomics.

1.9 Organelle proteomic – focus on chloroplasts

Plastids are indispensable plant cell organelles that performed many essential steps in plant cell metabolism. One of the characteristics is their ability to develop and differentiate into different plastid types, depending on their cellular and tissue environment. All different plastid types originate from undifferentiated plastid precursor organelles termed proplastids, which upon internal and external signals develop into e.g., amyloplasts in storage tissue, chloroplasts in photosynthetic leave tissue (Fig. 9) and chromoplasts in fruits and flower tissues. Less prominent plastid types encompass gerontoplasts, which are specialized aged chloroplasts that play a role in resource allocation, oleoplasts, which are oil storage plastids in e.g. olive, and etioplasts, which are the product of proplastid development in photosynthetic tissue in absence of light. The occurrence of solely undifferentiated proplastid is restricted to meristematic tissues and undifferentiated cells.
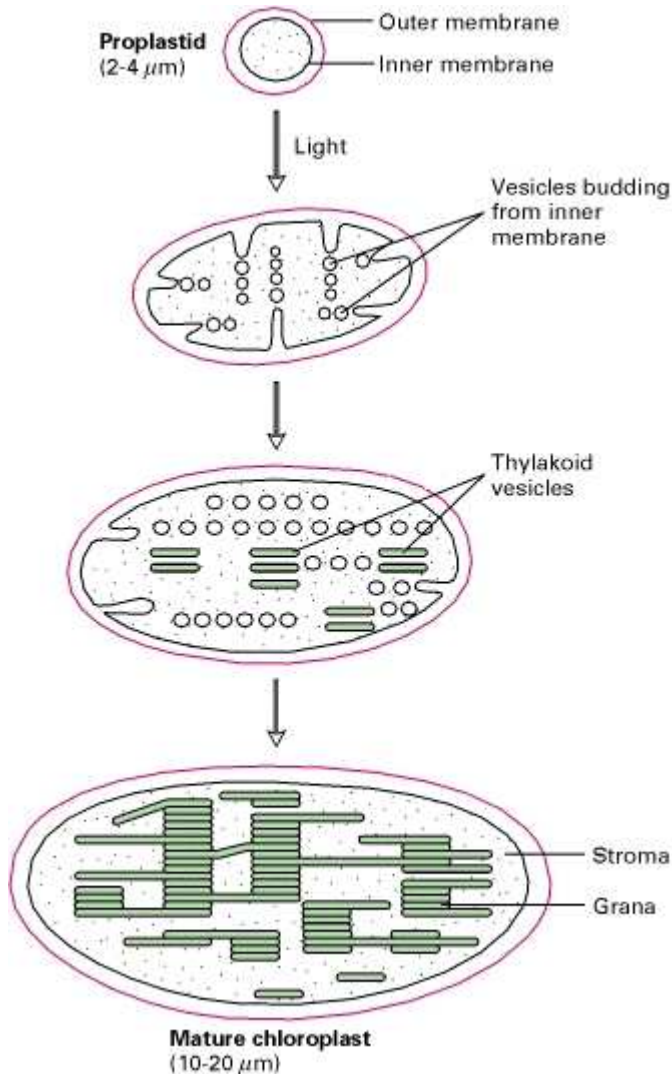
Figure 9. Formation of chloroplasts from proplastids. Light-induced budding of the inner membrane.

Because *Arabidopsis* does not develop sophisticated storage organs, flowers or fruits and the meristem is restricted to a few cells in the shoot apex, most plastid types are virtually excluded from *Arabidopsis* proteomics research. In contrast, *Arabidopsis* has been the organism of choice for the analysis of chloroplasts and is currently the only plant for which a truly comprehensive set of established chloroplast proteins is available. Furthermore it is now

known that *Arabidopsis* chloroplasts are not only important centers metabolic reactions but also that they are important signalling hubs that determine the expression of numerous nuclear encoded genes in retrograde signal cascades.

The predicted size of the combined proteome of all plastid types ranges from 2,000 to 3,500 proteins in *Arabidopsis thaliana*, representing about 7% to 12% of all predicted protein-encoding genes. However, only about 1,200 proteins are currently recognized as being plastid localized (see the Plant Proteome Database [PPDB] at http://ppdb.tc.cornell.edu). Comparing this experimental plastid proteome data set with the predicted plastid proteome showed that, in particular, plastid proteins involved in signalling and plastid gene expression and RNA metabolism are strongly underrepresented. There are several reasons why a significant percentage of plastid proteins has not yet been recognized:

(1) low abundance in chloroplasts (i.e. their detection is obscured by highly abundant photosynthetic proteins);

(2) specific expression in a certain plastid type other than chloroplast;

(3) only expressed under very specific conditions (developmental state, abiotic condition, or biotic challenge); or **(4)** too few ionizable tryptic peptides (e.g. transmembrane proteins with very short loops and tails or very small or basic proteins).

The early chloroplast proteome analyses were mostly conducted with density gradient isolated organelles. First, suborganellar compartments were mapped e.g. the thylakoid lumen, isolated thylakoid and envelope membranes and later on the chloroplast stroma and entire chloroplasts (reviewed in 67, 68). With the accumulated information from the latter mentioned analyses we now have a large catalogue of proteins that were identified from plastid preparations. Bioinformatics analyses with targeting

prediction tools on this proteins set suggested that the large number of these proteins cannot be predicted. As a matter of fact, with an increasing number of proteins identification, i.e. with increasing sensitivity of the analyses, more and more such proteins were identified that were considered "unexpected" in plastid because of the lack of recognizable targeting information, i.e. transit peptides. For example, Kleffmann and colleagues reported TargetP prediction sensitivities, i.e. rate of correct predictions of all true plastid proteins around 70% instead of 85% as determined by TargetP benchmark (69,70). Zybailov and colleagues identified more than 1325 proteins from isolated plastid, and sorted out 409 of these as putative contaminants (71). Rolland and colleagues established 1323 proteins from highly purified organelles (72). However, some of these proteins may not actually enter the plastid, but rather associate with plastid membranes from the cytosol, such as glycolitic enzymes, and targeting prediction software will miss a certain number of true chloroplast proteins, because plastid transit peptides do not have clear features and are thus difficult to recognize.

AIM OF RESEARCH

The target of this study is to find a protocol to isolate pure and active chloroplasts from Arabidopsis thaliana in order to analyze the impact of stress condition that affected proteins (deletion or overproduction) that can be functionally linked to photosynthesis thus providing information about their role at chloroplast level.

The protocol presented here is adapted from previously published protocols and improved with some tricks to optimize yield and purity.

A proteome study based on 2-D gel electrophoresis was performed in order to analyse the freezing stress response of *Arabidopsis thaliana* chloroplasts. Spot identification of interesting proteins was performed by nLC-ESI-MS/MS analysis. We chose *Arabidopsis thaliana* as model plant because of its importance for basic research in molecular biology and its capacity of cold acclimation and freezing tolerance. The study was more concerned with freezing tolerance than with cold acclimation, which is the ability to survive long- or short-term exposures at low but non freezing temperatures. Through a comparative analysis of protein patterns, we were interested in monitoring the overall changes in the protein complement after a sub-lethal exposure to -10 °C in cold acclimated plants and in non acclimated ones. Protein patterns were monitored after re-shifting plants to control conditions for a day, because the interest was to elucidate the later adaptations on the protein level following initial signalling events.

Low temperature is one of the most important factors limiting the growth, development and distribution of plants, adversely affecting the productivity and quality of crop plants. Thus, interest in understanding the molecular basis of freezing tolerance is driven by the desire to understand the mechanisms with which plants have evolved to tolerate environmental stress.

Chapter 2 Materials and Methods

2.1 Plant material and stress treatment

Preliminary analyses were performed in order to set optimal work protocol conditions and plant parameters for following analysis.

We used *Arabidopsis thaliana* ecotypes Col-0. Seeds were sown in plastic pots filled with a 1:1 mixture of commercial potting mix and fritted clay. Seedlings were grown in a controlled environment chamber at 23°C with a 16/8-h photoperiod and a photosynthetic photon flux density (PPFD) of about 150 μmol photons m^{-2} sec^{-1}. Plants were irrigated as necessary. Relative humidity was around 60-70% (Fig. 10). Cross experiments were performed using those parameters:

- **Plants age**
- **Cold acclimation treatment**
- **Freezing temperature**
- **Time of exposition to each temperature**

Each treatment was repeated at least three times. Untreated and stress treated plant samples were collected and used either immediately for analyses or frozen in liquid nitrogen and stored at –80 °C for further use. To evaluate freezing tolerance, plants were divided into two groups. Plants in one group were used to measure freezing tolerance without cold acclimation (NA) and were maintained at 23°C with a 16/8-h photoperiod. Plants in the other group (A) were transferred to a cold-acclimation chamber with exposure at 4°C for 7 d with a 16/8-h photoperiod. Whole plants were used to evaluate freezing tolerance and were placed in a programmable freezer. The temperature of the bath was set to 0°C and programmed to decrease to

the desired temperature with 1°C decrement in 30 min and maintained at that temperature for a determined time.
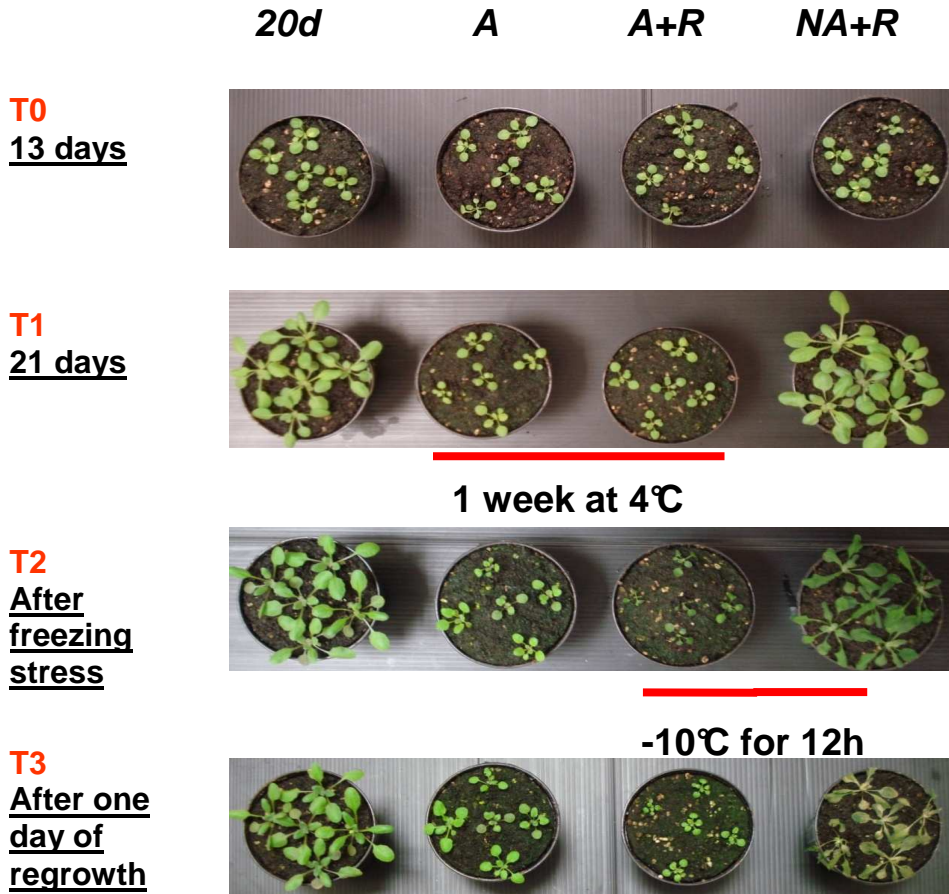
Figure 10. *A. thaliana* cultivated under different temperature. Plant status: 20-days-old *Arabidopsis* plants (20 d). Half of the plants were harvested and half were submitted to freezing treatment (-10°C for 12h, NA+R, T2) and were shifted back to control condition (T3). 13-days-old *Arabidopsis* plants (T0) were shifted to cold stress-conditions (A, T1). After a cultivation time of 1 week (4°C) half of the acclimated plants was harvested, and half were submitted to freezing treatment and shifted back to the control condition and then harvested (A+R).

2.2 Chlorophyll fluorescence

Maximum quantum efficiency of PSII (Fv/Fm) and quantum yield of electron transfer at PSII (Φ_{PSII}), were measured with a PAM 101 fluorimeter (Walz, Effeltrich, Germany). Fv/Fm, and Φ_{PSII} were calculated in this way:

$F_v/F_m = (F_m - F_o)/F_m$; $\Phi_{PSII} = (F_m' - F_s)/F_m'$. F_o is the initial fluorescence under non actinic light and F_m is the maximum Chl fluorescence from dark-adapted leaves, after a saturating pulse (in order to reoxidize the primary electron acceptor of PSII). F'_m is the maximum Chl fluorescence and F_s the stationary fluorescence during steady state illumination.

2.3 Pigment analysis

The composition of the pigments is analyzed after extraction with acetone 80% by RP-HPLC following in this case the method of Gilmore and Yamamoto (73): the sample is loaded on a reverse phase column (Spherisorb C18. 7.3 x 300mm) pre-equilibrated with buffer A. The chromatographic run is composed by 3 min flow with 90% Buffer A and 10% Buffer B, followed by a linear gradient to 100% buffer B in 9 min, followed by 3 min with 100% Buffer B and a linear gradient to 100% buffer A in 2 min followed by 4 min of 100 buffer A.

Buffer A

- Acetonitrile 86.8% (72 parts)

- Methanol 9.6% (8 parts)

- 0.1M Tris-HCl 3.6% (3 parts)

Buffer B

- Methanol 80% (4 parts)

- Hexane 20% (1 part)

2.4 Peroxidation level

Following the protocol of Vavilin *et al.,* 1998 (74) leaf disks were frozen and reduced to powder with a pestel and the pellet was mixed with 200 µl of sol. buffer (175 mM NaCl, 50 mM Tris-HCl pH 8, 0.01% butylated hydrotoluene in ethanol).

Then 200 µl of a solution of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid was added, followed by an incubation at 95° C for 25 min. Then, the reaction mixture was centrifuged at maximum speed for 20 min.

The MDA-(TBA) adduct was separated and quantified by HPLC. The analytical column and the HPLC apparatus were similar to those used for pigment analyses. The elution buffer was 65% 50 mM KH_2PO_4-KOH, pH 7, and 35% methanol. The time of chromatography was 12 min, with a flow rate of 0.8 mL/min, injected volume of 120 mL, detection at visible light (532 nm). The average retention time of the MDA-(TBA) adduct was 8 min. The levels of MDA were calculated using tetraethoxy-propane (Sigma-Aldrich) as a standard.

2.5 Chloroplasts isolation

The chloroplasts isolation method includes mechanical cell wall and membrane breakage, removal of cell debris and unbroken leaf tissue by filtration, collection of total cell chloroplasts by centrifugation and separation of intact from broken chloroplasts using a Percoll gradient.

In order to obtain a good preparation it is necessary to keep plants in the dark for 14- 16 hours, to avoid the formation of starch in the chloroplasts. Furthermore, it is important that leaves contain as little water as possible and for this reason we should avoid to water plants two days before the preparation.

The whole preparation must be carried out by using tools, reactants and rotors which have been cooled down to 4°C or which have been placed into ice.

- Cut and homogenize leaves in the presence of Grinding Buffer (GB) 1X (about 1 litre every 50 g) with politron. It is important to homogenize briefly, in order to avoid breaking chloroplasts (2-3 times for 10 seconds).

- Seeve the homogenized material through four layers of Miracloth.

- Centrifuge 6000g for 30 min,

- Discard the supernatant and gently resuspend the pellet again by using a brush and add 3-5 ml of GB 1X.

- Make a "step-gradient" with Percoll 80% and Percoll 40% into centrifuge tubes (in tubes/pipes for spin- dryer) of 50 ml.

First put the Percoll 80% solution (15 ml) on the bottom of the tube, then gently stratify 15 ml of Percoll 40% without mixing the two solutions. It is possible to put at most 4 ml chloroplasts suspension on each gradient, so two gradients are needed for a 100g sample of leaves.

- Centrifuge for 30 minutes at 3600 rpm in a swinging rotor. In this way intact chloroplasts form a band in the gradient at the interface between 80% and 40% of Percoll layers. At the end it should be possible to observe one pellet on the bottom and a green band (intact chloroplasts) between the two steps of the gradient. In the upper part of the gradient thylakoids can be recovered, but which must be further purified.

- Gently remove the Percoll 40% and suck out intact chloroplasts by using a pipette. Put the solution containing chloroplasts in a tube for SS34 and dilute with two volumes GB 1X.

- Centrifuge at 10000g for 1 minute to recover the chloroplasts which are on the bottom.

- Discard the supernatant and energically resuspend again the chloroplasts by using a pipette in 1 ml of hypotonic buffer. Transfer in eppendorf.

- Vortex and centrifuge at 13000 rpm for 15 minutes.

- At this stage the supernatant contains the proteins of stroma, while the pellet is formed by thylakoids.

- Resuspend again the pellet in 0.5 ml of hypotonic buffer and centrifuge at 13000 rpm for 15 minutes. In this way traces of stromatic proteins can be eliminated from the thylakoid fraction. These thylakoids are resuspended and kept in a buffer which contains 50% of glycerol, 10mM Hepes, 1mM EDTA.

- **Grinding Buffer (GB) 5X**: sorbitol 1.65 M, Hepes 0.25 M, EDTA 10 mM, $MgCl_2$ 5 mM, MnCl2 5 mM, brought to pH 8.0 with KOH and freshly prepared.

- **Hypotonic buffer**: Hepes 10 mM, DTT 5 mM, EDTA 1 mM, brought to pH 8.0 with KOH.

- **Percoll solution 100%**: dissolve 3 g of PEG 6000 and 1 g of Ficoll 400 in 100 ml of PERCOLL (Sigma, cat P1644) and autoclave. When cool, add 1g of BSA.

- **Percoll solution 80%**: 20 ml of GB 5X + 80 ml of Percoll solution 100% + 200 µl DTT 1M.

- **Percoll solution 40%**: 20 ml of GB 5X + 40 ml of Percoll solution 100% + 40 ml of water + 200 µl DTT 1M.

2.5.1 Estimation of the percent of intact chloroplast

- *Ferricyanide photoreduction*

The principle of the assay is based upon the inability of ferricyanide (an artificial electron acceptor) to cross the chloroplast envelope and react with

the electron transport system within the intact thylakoid membranes. Ferricyanide reduction, as indicated by the decrease in the absorbance at 410 nm, occurs only when ruptured chloroplasts are in the preparation. The percent of intact chloroplasts of the preparation is assessed by comparing the rates of ferricyanide photoreduction with and without osmotic shock of the chloroplasts.

For each of the following reactions, a volume of chloroplasts equivalent to 100 µg chlorophyll is used.

A. Without osmotic shock: mix chloroplasts with 4 ml of 1x CIB. Add 60 µl of 100 mM ferricyanide (prepared freshly in deionized water, final concentration 1.5 mM).

B. With osmotic shock: mix chloroplasts with 2 ml of water. Incubate for at least 15 seconds to allow for osmotic shock. Add 2 ml of 2x CIB and 60 µl of 100 mM ferricyanide.

  a. Place the tubes in a glass beaker filled with ice water

  b. Illuminate with a closely positioned 40 W bulb. Take a 1 ml sample before illumination, and then one every 2 minutes after illumination. Measure the absorbance at 410 nm using a spectrophotometer. Continue illumination for 6 minutes.

  c. Photoreduction of ferricyanide results in a decrease of the absorbance at 410 nm. Plot the absorbance at 410 nm versus the time. The rate of decrease in absorbance of each sample is the slope (ΔA_{410}/minute) of the graph. Calculate the slope of each reaction (A and B).

To calculate the percent of intact chloroplast, use the following formula:

$$((B-A)/B) * 100 = \% \text{ intact chloroplasts}$$

- *SDS-PAGE analysis*

A simple way to estimate the integrity of the purified chloroplasts relies on the use of an **SDS-PAGE analysis**, following the abundance of the major soluble protein (RbcL or RubisCO) from the stroma and the major thylakoid membrane proteins (LHCPs or Light Harvesting Complex Proteins).

2.6 Proteins extraction and quantification

Soluble proteins were extracted from chloroplasts according to Yang *et al.* (2007), with some modifications. Chloroplasts were ground in liquid nitrogen and homogenized with 1 mL of extraction buffer (5M urea, 2M thiourea, 40 mM Tris-HCl, 2% CHAPS, 50 mM DTT). The homogenates were centrifuged for 15 min at 15.000 x g. Supernatants were precipitated using TCA (15% v/v) containing 0.007% β-mercaptoethanol in acetone at -20°C for 2 h and then at 4°C for a minimum of 2 h. Samples were centrifuged at 4°C for 15 min at 14.000 x g, supernatant were discarded and pellets were washed twice with ice cold acetone containing 0.007% β-mercaptoethanol. Pellets were dissolved in a rehydration buffer (5M urea, 2M thiourea, 2% CHAPS, 50 mM DTT). Protein quantification was performed using Bradford-based assay kit assay (Bio Rad Hercules, CA), using bovine serum albumin as a standard.

2.7 Two-dimensional electrophoresis

Isoelectric focusing (IEF) of total proteins was performed using 18 cm-long immobilized pH gradient (IPG) strips, pH 3-10 non linear and pH 4-7. The protein sample was mixed with a rehydration buffer, 0.5% IPG buffer (v/v) of respective pH range and 0.002% bromophenol blue to a final volume of 340 µL and loaded onto the IEF strips. Samples were loaded onto IPG strips by passive rehydration. For analytical gels, performed to obtain the silver-

stained protein maps, 100 µg of protein sample was loaded. Instead 1 mg sample was loaded for the preparative gels stained with mass spectrometry (MS) compatible Coomassie brilliant blue (CBB) staining. IEF was carried out at 200V for 3h, 1000V for 1h, 2000V for 1h, 3500V for 1h and 35kVh using Multiphor II system (Amersham Pharmacia Biotech). Before running the second dimension SDS-PAGE, IPG strips were equilibrate twice in an equilibration buffer (6M urea, 30% glycerol (v/v), 50 mM tris-HCl, 2% SDS) for 15 min. The first equilibration was done using 1.2% DTT (w/v) in an equilibration buffer, while in the second equilibration, DTT was replaced by 1.5% iodoacetamide (w/v). SDS-PAGE was performed using 12.5% polyacrylamide gels at 15 °C using a Bio Rad Protean II XI (20 cm x 20 cm) vertical gel electrophoresis chamber. After completion of electrophoresis, gels were fixed and stained. The analytical gels were stained for image analysis with silver nitrate as described by Oakley *et al.* (1980, (75)). For MS analysis, the preparative gels were stained with CBB according to the manufacturer's instructions. Three independent biological replicates, each with four technical replicates were run for the analytical gels.

2.8 Silver staining

The silver stain is based on the ammoniacal silver/formaldehyde method of Oakley *et al.* (1980). This method involves preliminary glutaraldehyde treatment of the slab gel to fix proteins by cross linking. The pre-treatment also adds glutaraldehyde side chains to the proteins, increasing sensitivity since these groups are sites for silver deposition. The method is about 10 times more sensitive than Coomassie blue staining, depending on the protein, even though less protein is loaded. Generally, 50 ng of purified protein gives a highly visible spot. However, some proteins that are detectable with Coomassie do not stain at all with silver. Although very

sensitive, this method gives semi-quantitative rather than quantitative results. The linear range for plots of stain density versus ng protein varies from protein to protein with different saturation levels. Even so, silver staining is useful for computerized comparisons because spot values are normalized - expressed as a percentage of all spots combined. Values from duplicate gels are always averaged. This particular method of silver staining is not compatible with mass spectrometry fingerprinting but the patterns match well to a heavily loaded, Coomassie stained duplicate gel. Any spot present on the latter, no matter how faint, is within range for mass spectrometry identification minimal requirement. For silver staining of 2D gels, each 2D gel was soaked in a fixation solution of 40% methanol and 10% acetic acid, in water for 30 min and then washed in water for 10 min, followed by its sensitization in 2 % glutardialdehyde solution for 30 min. After rinsing eight times with water for 15 min, the 2D gel was stained for 30 min in silver nitrate solution. The silver nitrate solution was discarded and then the 2D gel was washed twice with water for 4 min. The 2D gel was developed in a developer solution with intensive shaking. After the desired intensity of staining was achieved, the developer solution was discarded and the reaction stopped in a solution of 5% acetic acid for 10 min (Tab. 1). The silver stained 2D gel was stored in deionized water at 4 °C until analyzed.

Step	Reagent	Duration
Fixing	40% methanol / 10% acetic acid	30 min
Wash	deionized water	1 x 10 min
Sensitizing	2% glutardialdehyde solution / 0.5M sodium acetate	30 min
Wash	deionized water	8 x 15 min
Silver	8g/l silver nitrate in 0.25% ammonia, 5% sodium hydroxide (1M)	30 min
Wash	deionized water	2 x 5 min
Development	0.1% formaldehyde (37%), 0.025% citric acid (2.3 M)	Up to spot appearance
Wash	deionized water	1 x 20 sec
Stop	5% acetic acid	10 min
Wash	deionized water	3 x 10 min

Table 1. Steps in Silver staining protocol. Silver staining is currently the most popular method for analytical purposes, which is 50 time more sensitive than coomassie blue staining.

2.9 Coomassie staining

Brilliant Blue G-Colloidal Concentrate Coomassie staining was done according to Sigma instruction. Each 2-D gel was soaked in a fixation solution for 30 min and then stained for 1-2 hs in the Brilliant Blue G-Colloidal Concentrate solution. The solution was discarded and then the 2-D gel was destained first with 10% acetic acid in 25% (v/v) methanol for 60 seconds and then in 25% methanol for up to 24 hours (Tab. 2). The 2-D gel was stored in water at 4 °C until analyzed.

Step	Reagent	Duration
Fixing	50% methanol, 10% acetic acid	30 min
Staining	75% Brilliant Blue G-Colloidal Concentrate solution, 25% methanol	1-2 hs
Destaining	10% acetic acid, 25% methanol	1 x 60 sec
Destaining	25% methanol	Up to 24 hs
Stop	50% methanol, 12% acetic acid	10 min

Table 2. **Steps in Coomasssie brilliant blue staining.** Preparative gels were stained with coomassie brilliant blue according to the manufacturer's instructions.

2.10 Computer analysis

Images were acquired using a ProXepress CCD camera system (Perkin Elmer). Computer-assisted 2D analysis of two to three recurrences of each gel was done by using PDQuest version 7.1 (BioRad) software. For the detection of spots, the area of interest was chosen by setting a crop polygon. Matching of protein/peptide spots and background subtraction were performed only partially automatically, whilst normalization was performed automatically by the software, without manual interference. Each spot in 2D gels corresponds to a protein or a polypeptide with or without a post-translational modification. We are aware that several spots may correspond to the same core polypeptide and for simplification we use the term 'protein'. Molecular weight of the proteins was calculated using the standard ladder applied to the gels and pI of the proteins were determined according to the pH value in the strips.

2.11 Protein identification by MALDI-TOF and nLC-ESI-MS/MS

Protein spots of interest were excised from gels, reduced, alkylated and digested overnight with bovine trypsin (Roche Diagnostics Corp). Aliquots of the supernatant (1 µL) were used for MS analysis by the dried-droplet

technique, using α-cyano-4-hydroxycinnamic acid as a matrix. Mass spectra were obtained with a MALDI-TOF Voyager DE-STR from Applied Biosystems/MDS Sciex. Ions were generated by irradiation with a pulsed nitrogen laser (337 nm UV, pulse duration 3 ns, pulse rate 3 Hz), and positive ions were accelerated and detected in the reflector mode. Instrument settings were: accelerating 20'000 V, grid 64%, guide wire 0%, delay time 200 ns, shots/spectrum 100, mass range 750-4000 Da and low mass gate 700 Da. Spectra were acquired via Voyager Control Panel 5.10 from Applied Biosystems. Once acquired, spectra were processed with Data Explorer 4.0 from Applied Biosystems and internally calibrated with trypsin autolysis products and matrix clusters. MALDI-TOF data led to extracted and manually curated peptide monoisotopic peak lists (deprived from trypsin and matrix clusters signals) that were searched, via in-house Mascot Sever 2.2.07, against the target database as detailed below except for mass tolerance for monoisotopic data that was set to 50 ppm and significance threshold of $p < 0.05$ set for the probability based Mascot Mowse Score.

For ESI analysis aliquots (5 µl) of tripsin digested sample were injected in a capillary chromatographic system Agilent 1100 Series equipped with Nano Pump, Iso Pump, Deaser and a 8 µl injection loop (Agilent). Peptide separations occurred on a 10-15 cm fused silica emitter used as analytical RP nano columm. The emitter was packed in-house with a methanol slurry of reverse-phase, fully end-capped 3 µm ReproSil-Pur 120 C18-AQ resin (Dr. Maisch GmbH), using a pressurized "packing bomb" operated at 50-60 bars. Mobile phases consisted of water with 2% acetonitrile, 0.1% formic acid (v/v; buffer A) and acetonitrile with 2% water, 0.1% formic acid (v/v; buffer B). A 55 min gradient from 8% to 80% buffer B at a constant flow rate of 200 µl/min was used for peptides separation. Eluting peptides were ionized by a nanoelectrospray ion source (Proxen Biosystems) and analysed on an API

QStar PULSAR (PE-Sciex) mass spectrometer. Analysis were performed in positive ion mode; the HV Potential was set up around 1.8-2.0 kV. Full scan mass spectra ranging from 350 to 1600 Da were collected, and for each MS spectrum, the two most intense doubly and triply changed ions peaks were selected for fragmentation (MS/MS range from 100 to 1600 Da). MS/MS spectra data files from each chromatographic run were combined and converted to mgf files using Mascot.dll (version 1.6b27) through Anayst QS 1.1 (Applied Biosystems) and searched (via Mascot Daemon 2.2.2 and in-house Mascot Server 2.2.07), first against a custom contaminant database (trypsin and common keratins partly derived from the cRAP collection), unmatched signals were then searched against the UniProtKB database. Mass tolerance was set to 200 ppm and 0.3 Da for precursor and fragment ions respectively. Searches were performed with trypsin specificity, alkylation of cysteine by carbamidomethylation and oxidation of methionine as fixed and variable modifications respectively, ion score cut-off set to 20, two missed cleavages were allowed for trypsin specificity, the quality MS/MS identifications was manually checked (Fig. 11).
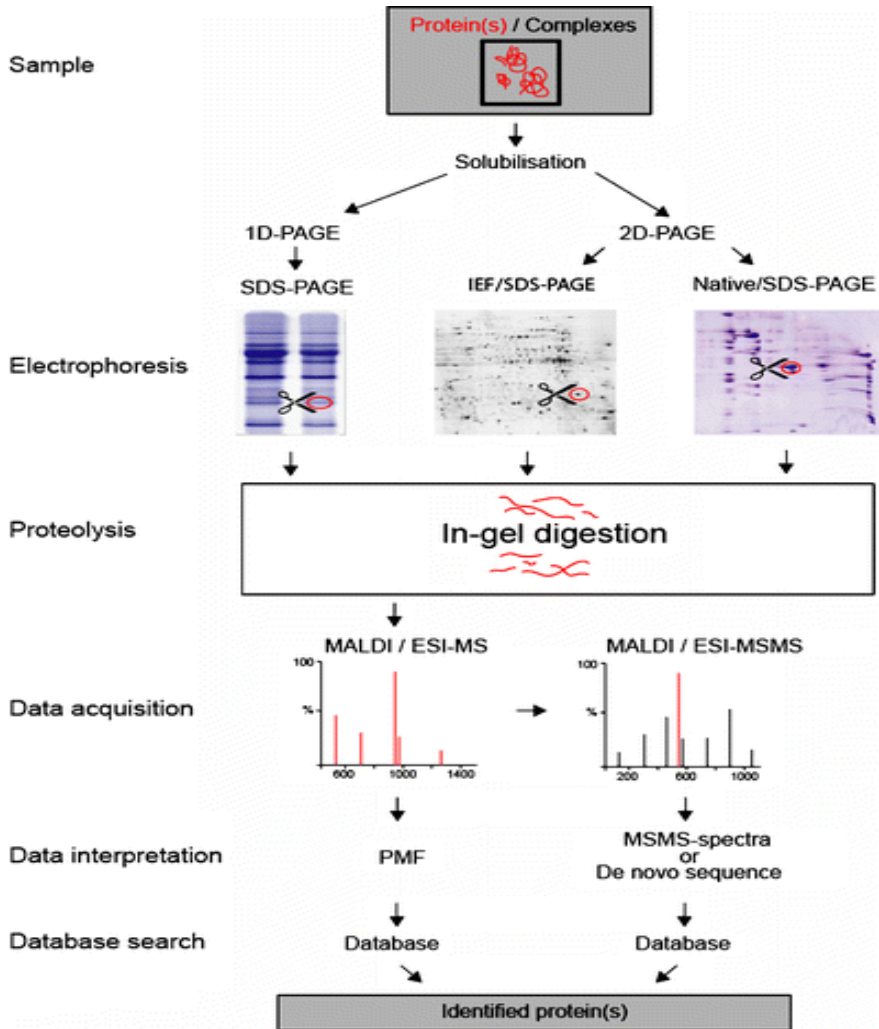
Figure 11. Typical workflow for sample processing, in-gel digestion, and mass spectrometry-based identification of a protein. The proteome of an organism or organelle is separated by 1D or 2D electrophoresis after solubilization of the protein or protein complexes. After the gel is stained, proteins of interest can be excised and processed by in-gel digestion. In-gel digestion results in production of peptides that are processed for mass spectrometric analysis by MALDI-MS or ESI-MS. The protein is identified by comparison of peptide mass fingerprint signals (PMF) or MS–MS spectra with proteome database entries, or the amino acid composition of the peptides is analyzed directly (de novo sequence). From Granvogl *et al.*, 2007 (76).
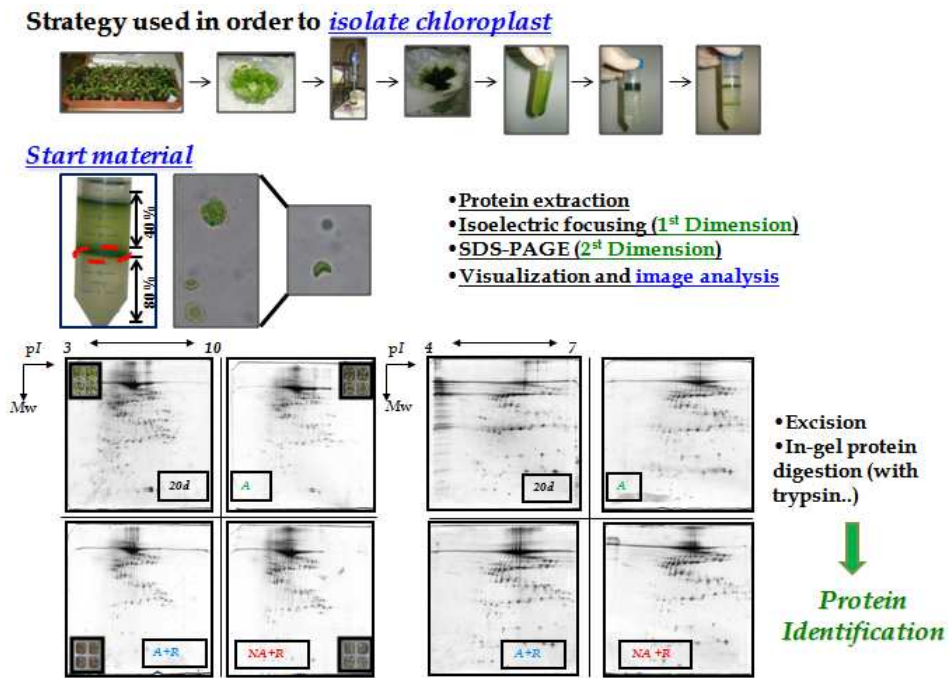
In summary (Fig. 12):

Figure 12. **Strategy used to purify intact chloroplasts from *Arabidopsis* leaves**. In order to obtain a good preparation it is necessary to keep plants in the dark for 14- 16 hours, to avoid the formation of starch in the chloroplasts. The whole preparation must be carried out by using tools, reactants and rotors which have been cooled down to 4°C or which have been placed into ice. Representative 2-DE profiles of chloroplasts proteins from *A. thaliana*. 100 μg proteins were loaded on non linear gradient 18-cm IPG strips with pH 3-10 and 4-7.

2.12 GO enrichment

Gene ontology (GO) term enrichment analysis to find statistically over- or under represented categories was performed with BiNGO 2.44 as a plugin for Cytoscape 2.8.0; the latest available ontology (obo 1.2 format) and *Arabidopsis* annotations files were downloaded respectively from the Gene Ontology and from the Gene Ontology Annotation (GOA) websites. The

whole *Arabidopsis* GOA annotation file was used as a reference set. Hypergeometric test, Benjamini & Hochberg false discovery rate correction and a significance level of 0.05 were chosen as parameters to visualize in Cytoscape the over-represented categories after correction. Due to the interdependency of functional categories in the GO hierarchy, it is very likely that not one category, but a whole branch of the GO hierarchy lights up as being significantly over-represented. In such cases the nodes which are furthest down the hierarchy were chosen to be reported.

2.13 Total RNA extraction and real time PCR

Total RNA was extracted from leaves harvested at the end of experiment. Electrophoresis using a 1% agarose gel was performed for all RNA samples to check for RNA integrity, followed by spectrophotometric quantification. Contaminating DNA was removed using a TURBO DNA-free kit (Ambion). RNA was then reverse-transcribed using an iScript™ cDNA Synthesis kit (Bio Rad laboratories). Genes corresponding to our proteins of interest were identified by searching for the relative Accession Number in the UniProtKB. Expression analysis of these genes was performed by real-time PCR using an ABI Prism 7300 sequence detection system (Applied Biosystems). Quantitative PCR was performed by using 20 ng cDNA and iQ™ SYBR® Green Supermix (BioRad laboratories), according to the manufacturer's instructions. Expressions of Ubiquitin were used as endogenous controls. Relative expression levels were calculated using Genorm (http://medgen.ugent.be/jvdesomp/genorm). For the list of the primers used see Table 3.

Tab 3: List of primer used for RT-PCR

Spot No	UniProtKB Acc. No	Gene	Primers sequenze 5′-3′
		Ubiquitin	Forward: ATGTGGTTGGAGTCATGGCAAGGA Reverse: GGAGAAGTTGCAGTCTCAAAGC
2309	P0CJ48	AT1G29910.1	Forward: AAAGTTTCAATGGCCGCCTCCAC Reverse: ATGGACCCTTTGGCTTGGCAAC
2309	Q9T0L1	AT4G13400.1	Forward: TTTGGTTGGAGTCATGGCAAGGAG Reverse: ACCCTTCAGCATGTCAAGCTTCC
2309	Q9XF89	AT4G10340.1	Forward: GCCGTAGTTGCTGAGGTTGTTC Reverse: AGCTTGTCCTCGAAATCCAATCCG
2402	P10896	AT2G39730.1	Forward: AGACCGTATCGGTGTCTGCAAG Reverse: CCCTCAAAGCACCGAAGAAATCG
2402	P23321	AT5G66570.1	Forward: ACGTGAAGAACACTGCCGCTTC Reverse: TCTCCGGCTTGCTCTTTGTCAC
2523	Q9FWW5	AT1G12150.1	Forward: CGGAGAAGTTGCAGTCTCAAAGC Reverse: TTTCCTTGTCGCAAACGCTCTC
2523	Q43127	AT5G35630.1	Forward: GGTGAAGTTATGCCTGGACAGTGG Reverse: AGCACACCAAACATGATCACCTG
2523	P50318	AT1G56190.1	Forward: AAGCTCTGGATACCACGCAGAC Reverse: CTAGTTTATTCGCTACCGCCTCAG
2526	Q9C7F1	AT1G28020.1	Forward: TTCATGGTTCATGTCCGCATCG Reverse: CCACTGTTCTAGCACCGGAATG
2527	P17745	AT4G20360.1	Forward: ATGGTTATGCCCGGTGATCGAG Reverse: ACAAGCCACCGGCACAATAAGC
3311	O65282	AT5G20720.1	Forward: TTGCCGTGGGTGAAGGAAGAAC Reverse: TTGTGCTCCAGTAGGGACAGTG
3518	P19366	ATCG00480.1	Forward: ACATTGCTAAAGCTCATGGTGGTG Reverse: TTTCCTTCACGAGTCCGTTCGC
3518	O81016	AT2G26910.1	Forward: GGCTTCATGATCCCATACAAGAGG Reverse: ATAGACCGTACAGTGTCCAAGCC
3518	Q9M7Y4	AT3G06780.1	Forward: TCTTTCGCCGATTCCGTTGTTG Reverse: TCGCCGATGCTTGAACGATTCC
3518	O03042	ATCG00490.1	Forward: AGGAACTTTAGGCCACCCTTGG Reverse: TGCTTCCAGAGCTACTCGGTTG
4314	Q42029-2	AT1G06680.1	Forward: TGGTCACTCCTACCGACAAGAAG Reverse: CATTGTTGTCAAAGCCTCCCTCAG
4315	P34791	AT3G62030.1	Forward: GTGTTAAATCCATGGCTGCTGAGG Reverse: CCCATCACAATTCTGCCAGCAAC
4316	Q547G3	AT1G26630	Forward: TGGCACCAAGGATGATCTCAAGC Reverse: TCATCGAATCCAAGCCTCATCTGG
3609	Q42276	AT1G06950.1	Forward: AGAACATTTCATCGGCACTCAGC Reverse: CGACAGATGCCAAACTCTTCGC

Table 3. **Genes corresponding to our proteins of interest were identified by searching for the relative Accession Number in the UniProtKB**. Expression analysis of these genes was performed by real-time PCR using an ABI Prism 7300 sequence detection system (Applied Biosystems).

Chapter 3 Results and Discussion

3.1 A developed method for extracting total chloroplast proteins

A principal criterion for organelle proteome is the purity of the compartment to be analyzed. Indeed, the subcellular proteome integrity largely depended on how we can purify the isolated compartment away from other cellular contaminants. In this study, the chloroplasts were isolated from *Arabidopsis thaliana* leaves according to Salvi *at al.* (2011, (77)). The integrity of the isolated chloroplasts was subsequently analyzed by a microscope. The *A. thaliana* chloroplasts appear round and bright green surrounded by a bright halo of light (Fig. 13). Almost no broken chloroplasts were observed.
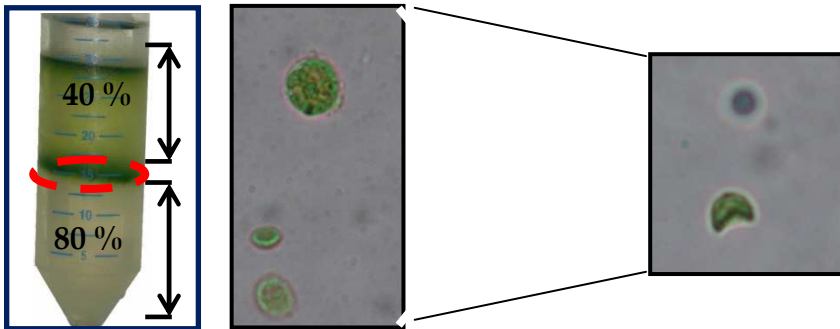

Figure 13. Intactness. Intact chloroplasts form a band at the interface of Percoll step gradient (red circle).

In addition to morpholological analysis by microscopy, the integrity of the purified chloroplasts can be confirmed performing the ferricyanide test for intactness (78). A 75% intactness is usually achieved for chloroplasts isolated using the Salvi protocol.

Contamination with non-chloroplast proteins was monitored by measuring the activity of catalase as a peroxisome marker in the chloroplast fraction.

The crude extracts showed high catalase activity, whereas the purified chloroplast fraction did not show any significant catalase activity (Fig. 14).
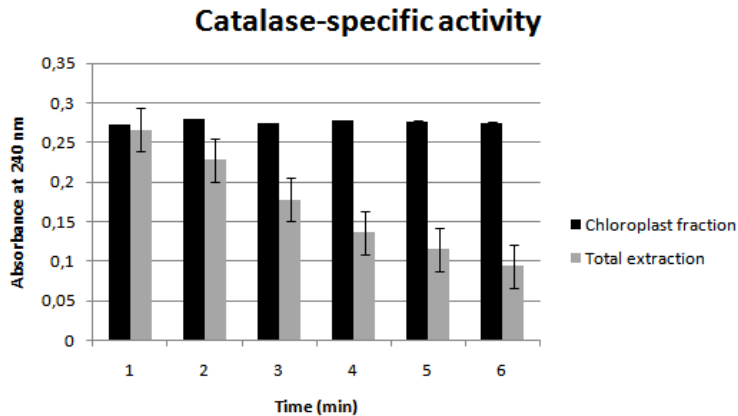

Figure 14. Determination of catalase-specific activity in the chloroplasts of *A. thaliana*. The total extraction prepared from *Arabidopsis thaliana* leaves for catalase activity was used as a positive control.

Beside purity, the physical integrity of the chloroplasts is another important prerequisite for analyses that aim to monitor their entire proteome. A simple method to estimate the intactness of isolate chloroplasts is to quantify the ratio of major soluble stroma and thylakoid proteins by SDS-PAGE. A high ratio of a large subunit of ribulose 1,5-bisphosphate carboxylase (RbcL, localized in the stroma) to the light harvesting complex proteins (LHCPs, bound to thylakoid membranes) was observed in chloroplasts fraction. This method relies on the demonstration that soluble proteins from stroma are not lost during the purification procedure. A protein profile obtained from pure and intact chloroplast preparations reveals that the amount of RbcL is higher than the amount of LHCPs. When broken chloroplasts are analyzed, and due to the loss of soluble proteins resulting from the breakage of the limiting envelope membranes, the ratio of RbcL/LHCPs is strongly reduced (Fig. 15).
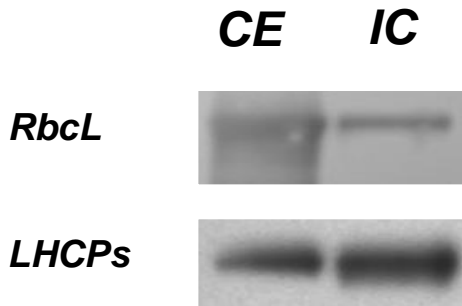
Figure 15. **SDS-PAGE analysis of Percol-purified chloroplasts from** *Arabidopsis*.
Fractions (15 μg protein) were analyzed on a 12% SDS-PAGE followed by
Coomassie-blue staining. Abbreviation: CE: crude extract, IC: intact chloroplasts.

These results, altogether, suggest that the isolated chloroplasts were highly
purified and had no appreciable level of other contamination.

In addition to the enrichment of plastidial proteins, this procedure is also
suitable for the investigation of plastidial lipids, pigments or other
metabolites.

3.2 Acclimation of whole plants to low temperature: pigments and photosynthetic
characteristics

- To be sure to work only with plants surviving the freezing treatment
  and since the interest was to investigate the late events of
  adaptations at the protein level following initial signalling events, we
  chose to perform proteomics analysis only on plants which recovered
  for a day at control condition (NA+R: 20 days-old plants, non
  acclimated, treated at -10 °C for 12 hours and collected after one day
  of regrowth at control condition; A+R: 20 days-old plants,
  acclimated, treated at -10 °C for 12 hours and collected after one day
  of regrowth at control condition).

In a photosynthetic organism, the light energy absorbed by chlorophyll (Chl) can be divided into three parts: that utilized photochemically to drive photosynthetic electron transport, that dissipated non-photochemically as heat and that emitted as fluorescence (79). These processes are competitive, and changes in Chl fluorescence may reflect alterations in photosynthetic functions (80). Abiotic stresses can directly or indirectly affect photosynthetic traits in leaves and change their Chl fluorescence properties (81). When plants are exposed to freezing stress, leaf metabolism is severely inhibited, and photo-damage to PSII happens unavoidably. The proportion of absorbed light utilized in photosynthetic electron transport decreased and the decrease was more pronounced under low temperature. Thus the freezing-shocked plants (A+R and NA+R) need to overcome excess energy when they are in recovery growth, in particular under high irradiance, to avoid photosynthetic apparatus and metabolic processes to be severely disturbed. On the other hand, it has been well documented that light, via photosynthetic process, provides the energy necessary for the induction of cold tolerance in plants.

Tab 4. Chlorophyll fluorescence parameters

Chlorophyll fluorescence		*dark* Fv/Fm	*light* *ΦPSII*	*light* ETR
T1				
	20d	0,785 **a**	0,718 **a**	*18,109 a*
after acclimation	**A**	0,770 **a**	0,678 **b**	*7,285 g*
T2				
	20d	0,765 **a**	0,695 **a**	*17,136 b*
	A	0,739 **b**	0,662 **b**	*16,785 c*
after freezing	**A+R**	0,690 **c**	0,284 **d**	*16,780 c*
	NA+R	0,381 **d**	0,157 **e**	*2,047 d*
T3				
	20d	0,773 **a**	0,696 **a**	*17,653 b*
	A	0,749 **b**	0,707 **a**	*18,436 a*
after recovery	**A+R**	0,662 **c**	0,539 **c**	*13,790 e*
	NA+R	0,051 **e**	0,026 **f**	*0,594 f*

Table 4. Changes in the quenching of excitation energy in the antennae of PSII can easily be estimated using modulated chlorophyll fluorescence. Each value is the average of 15 measure. The values are the average of 15 measurements.

In the **dark** acclimated leaves, the quantum yield of PSII was measured as the ratio of variable fluorescence to maximum fluorescence (Fv/Fm):

$$Fv/Fm = (Fm - Fo) / Fm$$

where:

- Fo, is the minimum fluorescence, emitted from the leaf when it is irradiated only with the modulated light of low irradiance and when the primary acceptor is fully oxidized,

- Fm, F_m is the maximum Chl fluorescence from dark-adapted leaves, after a saturating pulse,

- Fv / Fm represents the maximum quantum efficiency, when all the PSII reaction centers are open. Under optimal conditions (for plant control, 20d), the plants have reached the maximum value of Fv/Fm which is about 0.78 (see in Tab. 4).

In the **light** acclimated leaves, the following values were determined: the quantum yield of PSII (ΦPSII) and the apparent speed of the photosynthetic electron transport (ETR).

The value of ΦPSII was determined as:

$$ΦPSII = ΔF / F'm = (F'm - Ft) / F'm,$$

where :

- Ft is the fluorescence under actinic light and

- F'm is the **maximum** fluorescence under a saturating pulse.

Changes in values of Fv/Fm can be due to different causes. In general, the parameter ΦPSII decreases due to photoprotection mechanisms (dynamic photoinhibition), but could also represent the damage to PSII (chronic photoinhibition). The efficiency with which absorbed light energy is converted into chemical energy, is indeed subjected to regulation: it consists in modulating the rate of dissipation of heat in the form of radiation absorbed, so that the density of excitation energy in antenna of PSII is proportional to that required to drive the photosynthetic electron transport, which in turn depends on the reaction rates of assimilation of CO_2. The

decrease in Fv/Fm is instead caused by chronic photoinhibition, since it is a decrease in maximum quantum yield of PSII that occurs in leaves acclimated in the dark, when the photosynthetic electron transport is stopped and photoprotection mechanisms are, for most, disabled: it follows that, under these conditions, a low efficiency of PSII was due to a damage of the photosystem (and to a lesser extent to the activation of mechanisms of photoprotection more persistent).

In summary:

- Exposure of plants to the period at 4 ° C lowers very little Fv/Fm, so that temperature does not cause chronic photoinhibition.

- Fv / Fm decreases significantly when plants acclimated are then subjected to freezing and decreases very slightly in the subsequent recovery. Acclimatization is therefore of vital importance in preventing stress from freezing. In plants not acclimated, freezing leads to severe chronic photoinhibition and subsequent recovery, the values of Fv/Fm are even lower, when the plants seem to experience a high mortality.

- Of course, under actinic light all plants suffer a decline in ΦPSII, and this is largely due to photoprotective mechanisms (non photochemical quenching). The strongest differences are recorded in plants subjected to freezing, but with substantial differences, depending if plants were or not previously acclimated. Indeed, the difference between Fv/Fm and ΦPSII in plants acclimated assumes the highest value among all those observed, but this is mainly due to dynamic photoinhibition. There are two data supporting this interpretation: the apparent speed of the electron transport, which is high (16.780) and similar to that of plants not subjected to freezing and the high value of Fv/Fm (0.690), demonstrating that the damage to PSII is negligible. In non-acclimated plants, the gap between Fv/Fm and ΦPSII is lower, however it is evident that these plants are subjected to conditions of chronic

photoinhibition, as indicated by low values for both the apparent velocity of the electron transport (2.047) and for F_v/F_m (0.381) (as reported in Tab. 4).

The values of F_v/F_m measured decreased during the period of acclimation to low temperature and then returned to baseline values during the recovery period (as observed from T1 to T2 for plants acclimated at 4°C, A). Moreover this value was higher in control conditions and acclimatization (with or without exposure to freezing) because most of the absorbed light has been used in photochemical reactions, but was lower in plants exposed to freezing without previous acclimation, in which most part of the absorbed energy was dissipated by non-photochemical processes (Fig. 18, (82)).

With regard to the recovery under standard temperature (23°C), plants previously acclimated at 4°C recovered more rapidly and to a greater extent in terms of quantum yield of PSII, compared with non-acclimated, which show a irreversibility of the process (chronic photoinhibition). In acclimated plants, the capacity of non-photochemical quenching is instead increased, and it persists well beyond the stress during the whole period of recovery at 23°C, showing that in plants subjected to freezing stress, mechanisms of energy dissipation through the xanthophylls cycle are induced and remains active even after one day of recovery, T3 (Tab. 4).

Analysis of the results suggests that the cold stress has affected the efficiency of PSII, causing a decrease in quantum yield of PSII values (ΦPSII and F_v/F_m). It is important to remember that these two parameters are influenced not only by environmental factors to which plants are subjected, but also by physiological factors of the plant, such as age of the leaf and the health of the plant. The incidence of variability of these factors was mitigated by choosing for the measurements leaves of the same age and plants subjected to the same conditions of light (*light history*) and that, if subjected to freezing stress, apparently showed the same degree of suffering.

The fluorescence measurements can provide evidence on the ability of a plant to tolerate environmental stresses and the degree of damage to the photosynthetic work of these stresses. The lower values of Fv/Fm, which has been found in plants subjected to freezing stress indicates a state of chronic photoinhibition, which is transient in plants previously acclimated at 4°C, and persists in non-acclimated.

The response of the photosynthetic apparatus to alteration in the environment involves characteristics and well-documented alterations in the composition of thylakoid membranes that include changes in the contents of xanthophylls and the LHCs that bind them (83) as in our case. Chilling treatment of *Arabidopsis* is known to induce a particularly strong abiotic stress response that includes an increase in the xanthophylls cycle pool size and increased de-epoxidation (84).

Moreover dissipation of excess light energy during photosynthesis involves several photoprotective mechanisms, which are collectively referred to as non-photochemical quenching (NPQ). The predominant component of NPQ is referred to as energy-dependent quenching, or qE, and it is rapidly reversible and correlated with zeaxanthin (Z) formation (Fig. 18). Zeaxanthin appears to have a role in establishing tolerance to photooxidative stress by an NPQ-independent process, which protects the lipids of the thylakoid membrane from oxidative damage.

A very stable type of NPQ found in plants acclimated to low temperature shows a Zea-dependent feature called 'cold hard band'; in evergreen plants, this mechanism allows photosynthetic machinery to withstand freezing conditions over winter, thus maintaining a permanently photoprotected qI state of aggregated LHCII (85).
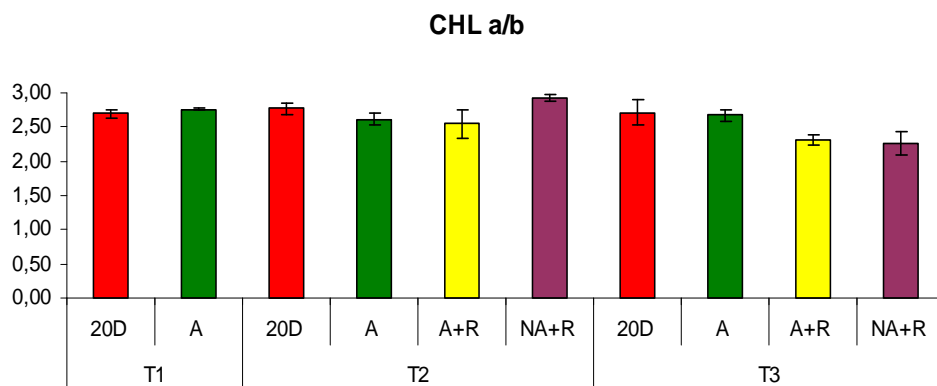
CHL a/b

Fig. 16. **Chlorophyll a/b ratio.** Modification of Chl a/b ratio in leaves subjected to the treatments described in Fig. 8. Its decrease suggests a modulation of the number of Chl a binding Lhc proteins per core complex.
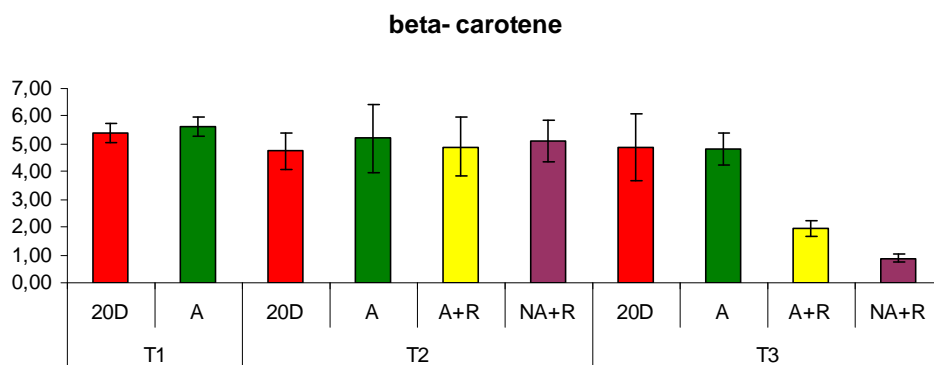
beta- carotene

Figure 17. **Beta-carotene amount**. Decrease in β-carotene observed after freezing treatment (T3). This decrease is in agreement with the decrease in Chl a/b, suggesting that the damage caused by low temperature affects the core complex in photosystem and in particular those proteins binding core complex (like CP26, as in our case, and CP29). The values for this graph and the following graphs (Fig. 18, 19, 20 related to pigments content) are percentages values, considering the chlorophyll content as reference (Chl = 100).
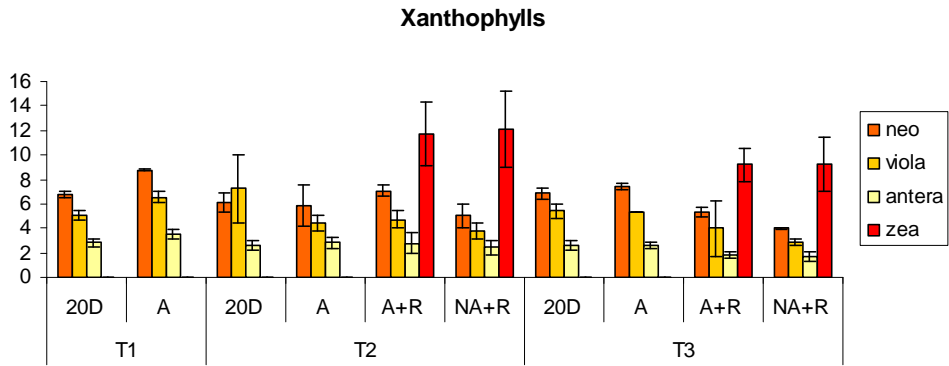
Xanthophylls

Figure. 18. **Beta-xanthophylls content.** The graph shows the decrease in content for neoxanthin (neo), violaxanthin (viola) and anteraxanthin (antera) after freezing treatment (T2), which is maintained after one day of regrowth at control conditions (T3). In the main time, we can notice the accumulation of zeaxanthin (zea) for samples undergo to freezing temperature (A+R and NA+R, T2), still present at T3. In fact, plants exposed to strong light (as in our case, with the effect of temperature in perceive light) convert violaxanthin into zeaxanthin, which increases their photoprotection ability. The values are percentages values, considering the chlorophyll content as reference (Chl = 100).
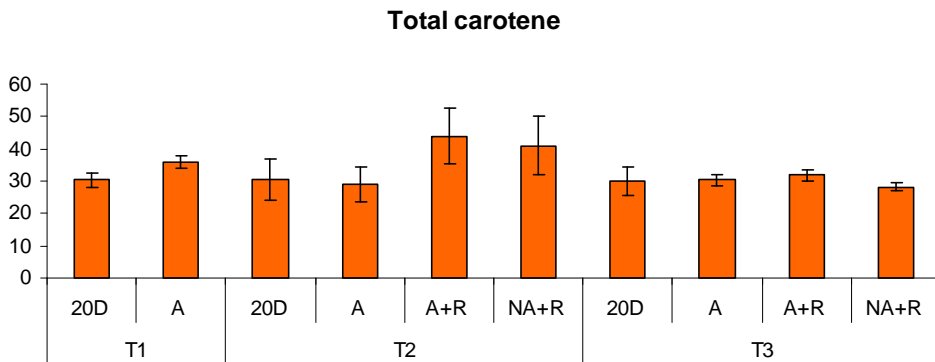
Total carotene

Figure. 19 The total amount of carotene increases after freezing treatment (T2). The values are percentages values, considering the chlorophyll content as reference (Chl = 100).
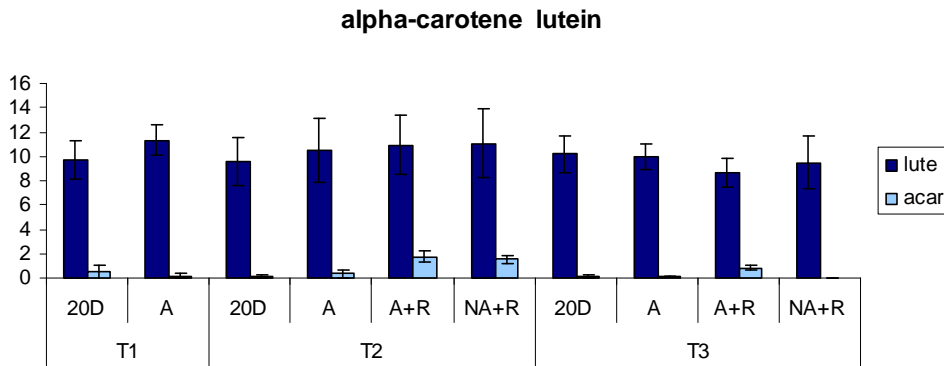
alpha-carotene lutein

Figure 20. **Alpha-carotene and lutein content.** Plants undergo to freezing treatment accumulate α-carotene (acar) and this lead to an increase of total carotenoids. If we look at lutein level (lute), this xanthophyll is constant among different samples under analysis; so we can speculate that the lutein observed in T3 for A+R and NA+R plants could have derive from α-carotene accumulated in T2. The values are percentages values, considering the chlorophyll content as reference (Chl = 100).

Moreover we notice that the total amount of carotenoids increase after freezing treatment (T2) (Fig. 16), and at the same time there is an accumulation of α-carotene in T2 (Fig. 17). The α-carotene accumulated at T2 may generate lutein at T3. But the fact that no increase is observed in lutein at T3 may be associated with an exaggerated response to chilling (86).

This might suggest that cold could stimulates a preferential pathway (which goes from lycopene to α-carotene), because while the β-carotene decrease (red arrow) along with beta-beta xanthophylls (red arrows), α-carotene increase (green arrows) and, in this way lutein remains constant between samples and during time (Fig 18).

An alternative possibility: beta-carotene may go down because it is metabolized when PS are affected by freezing, and the system uses alpha to compensate. While alpha is normally not incorporated in PS and thus converted.
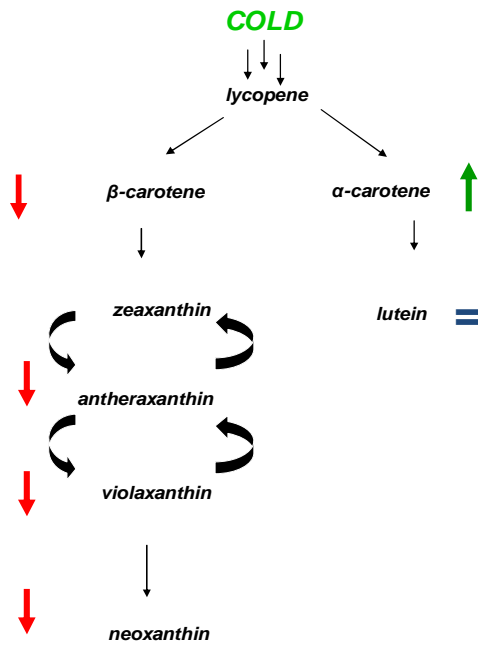
Figure 21. Hypothesis of carotenoids pathway mediated by cold.

The amplitude of xanthophyll's photoprotective effect, particularly of zeaxanthin and lutein, strongly depends on their binding to Lhc proteins.

3.3 Proteomics results: proteins differential expression

Because of its adverse effects on productivity, low temperature has been regarded as a major stress for crops and its negative effects have been studied extensively. Cold stress has been discovered to change several metabolic pathways and structural processes. We sought to insight into the survival process by inventorying the proteome during a few days of a stressful, but non-lethal, cold treatment.

To detect differentially expressed proteins associated with freezing tolerance, with or without previous acclimation, 2-DE patterns from control and the corresponding stress treatment were compared. Extracts were

prepared from chloroplast of *Arabidopsis thaliana* control and treated plants and proteins resolved by 2-D gel electrophoresis as described in the Materials and methods. To ensure the reproducibility of protein patterns, at the beginning we resolved protein samples on pH 3-10NL (18 cm) IPG gel strips, performed in triplicate biological repeats. In Fig. 22 , a representative image from a silver stained 2-DE gel is shown. As already known, silver staining provides generally the best spot staining sensitivity, but this staining is not compatible with further identification analysis by mass spectrometry. For this purpose we used colloidal Coomassie G 250, but the gel staining in 3-10 pH range was too low to permit discrimination of differentially expressed protein spots, even if a greater amount of protein was loaded (1000 µg). Thus, to improve the separation and resolution of proteins we applied the zoom-in gel approach and we used a pH range of 4-7 (Fig. 23).
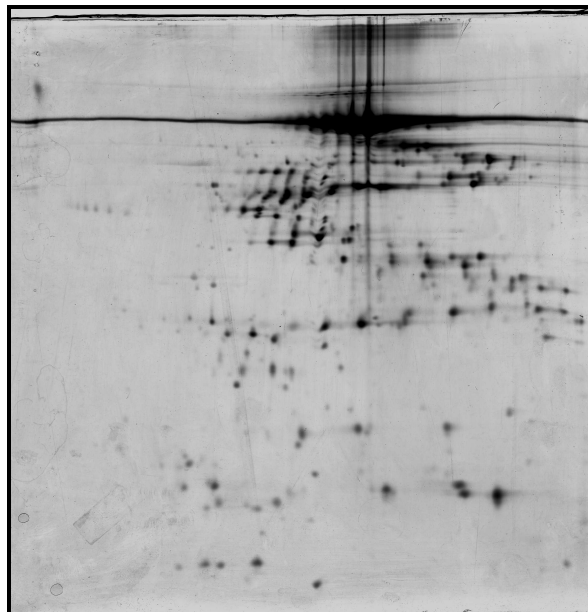

Figure 22. Reference 2-DE map of *Arabidopsis thaliana* chloroplast from 20 days-old rosette grown at standard condition. Gel staining with Silver, pH 4-7.
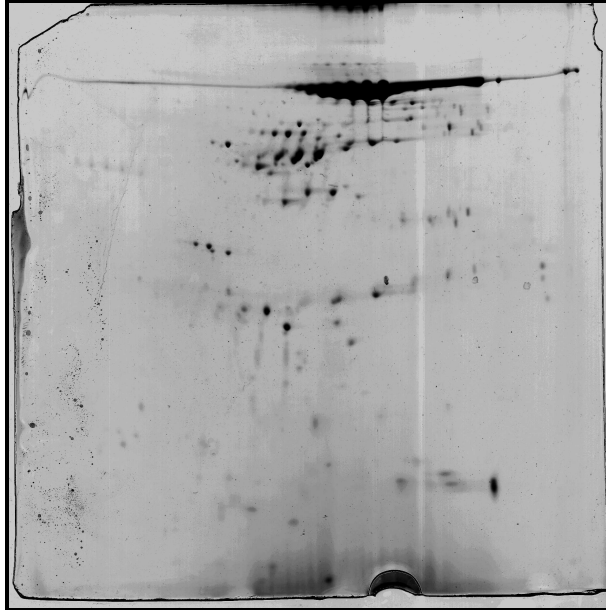
Figure 23. Reference 2-DE map of *Arabidopsis thaliana* chloroplast from 20 days-old rosette grown at standard condition. Gel staining with Coomassie, pH 4-7.

Computer-assisted 2D gel differential analysis was performed by using the PDQuest version 7.1 software (BioRad), to compare differential protein expression between control and treatments. The protein spots resulted to be differentially expressed from the image analysis were chose for protein identification.

The spots marked on the 2D-gels in Figures 24 were excised, submitted to tryptic digestion and mass spectrometry analysis as described in Material and Methods.
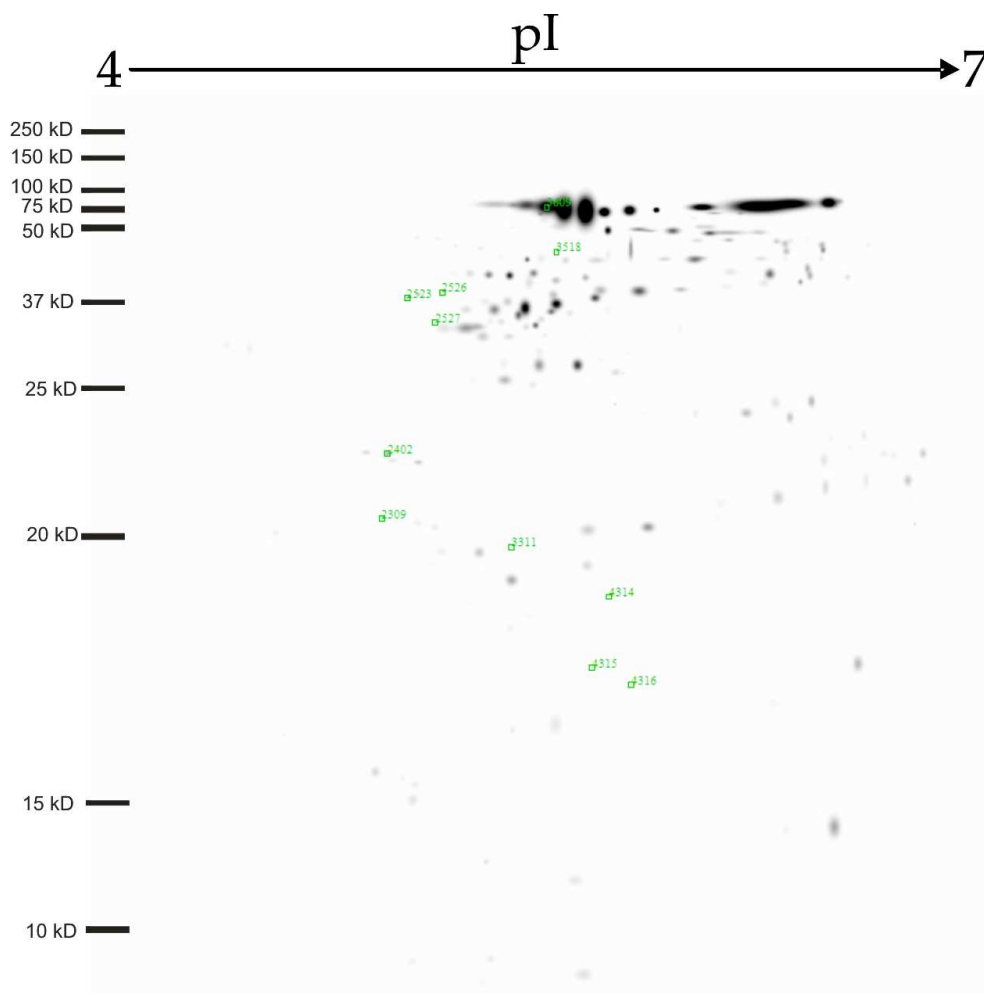
Figure 24. Representative 2-DE map of *A. thaliana* chloroplast. A total of 1000 μg of protein was extracted and separated by 2-DE. The differentially expressed proteins are numbered on the gels.

A total of 50 spots were analyzed, among these, 30 were successfully identified corresponding to 22 different proteins.

As expected, the major part of identified proteins (see Table 5- 6 and Fig. 27) are related to photosynthesis (Fig. 25A) but also to carbon and nitrogen metabolism.
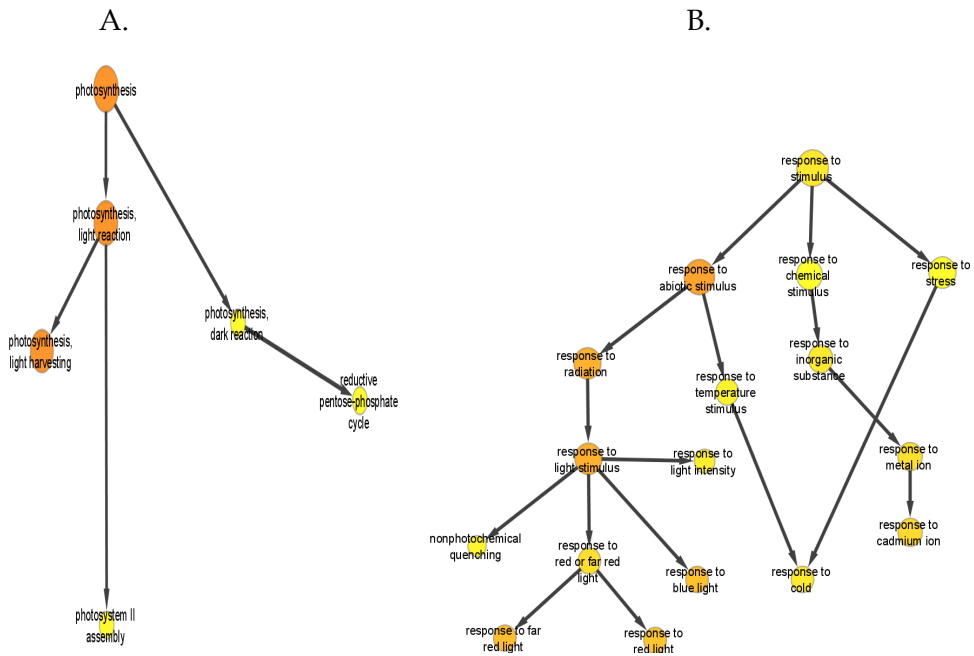
A.   B.

Figure 25. Biological process in which the identified proteins are involved, according to Gene ontology annotation. Graphical depiction of the over-represented GO terms with parent-child relationships and p-value colour coding of each node as obtained after statistical analysis. DR: Defense response; P:Photosynthesis related GO enrichment: Gene ontology (GO) term enrichment analysis to find statistically over- or under-represented categories was performed with BiNGO. A) GO: Photosynthesis; B) GO: Response to stimulus.

If we look at the gene ontology related to *Response to stimulus* (Fig. 25B) we can observe: response to cold, but also response to light and this result could be linked with the choice of harvesting plants after one day of regrowth at control condition. In fact, we know that plants perceive light in a different way if they are exposed to different temperatures. For this reason we investigated the level of lipid peroxidation (Fig. 26), which is higher for NA+R plants at T3.
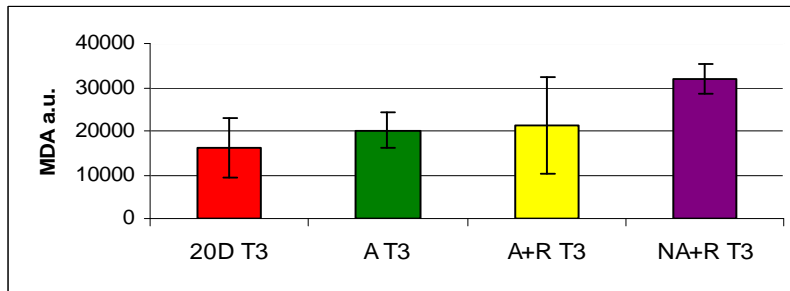
Figure 26. Lipid peroxidation in samples under analysis. Freezing treatment markedly increased lipid peroxidation in NA+R sample, indicated by augment of MDA level. Pre-treatment of plants (1 week at 4°C) attenuated this increased in lipid peroxidation in A+R, as indicated by a reduction by a reduction of MDA level. (n° = 6 in each group).

The physiological response. Interestingly some differentially expressed proteins appeared to be the products of degradation because their observed Mr values were much smaller than theoretical ones. A particular case was the photosynthetic proteins, including Rubisco large subunit (RcbL, spot 3518, Fig. 27r), Rubisco activase (RbcA, spot 2402, Fig. 27d) and photosystem II oxygen-evolving complex (spot 4314, Fig. 27t). All together 7 identified spots corresponded to the same protein, RcbL, which is the most abundant protein in leaves.

Photosynthesis

Photosynthetic carbon metabolism is initiated by Rubisco, which combines CO_2 with ribulose 1,5-bisphosphate (RuBP) to form two molecules of phosphoglyceric acid. The concentration of Rubisco, the carboxylating enzyme of the Calvin cycle, is generally high. For example, it accounts for 50% or more of the total protein in plant leaves, and its concentration within chloroplasts is extremely high (ca. 0.2 g/ml). Rubisco consists of 8 large (L)

and 8 small (S) subunits arranged as 4 dimers. Special proteins that modulate protein folding assist the formation of the L8S8 type of rubisco in a process that uses ATP. These proteins, called chaperonins, modify the non-covalent interactions of target proteins and bring about the active conformation of the enzyme. The changes in the activity of Rubisco are caused by "activation" by carbamoylation of a lysine residue and Mg^{2+}-binding in the active site. Activity sufficient for high rates of photosynthesis and growth at atmospheric concentrations of CO_2 depends on the activity of another protein, Rubisco activase (87,88). Rubisco activase is a nuclear-encoded chloroplast protein that is required for the light activation of Rubisco in vivo. Activase physically interacts with Rubisco, catalyzing ATP hydrolysis and facilitating the release of ribulose-1,5-bisphosphate and other tight-binding sugar phosphates from the active site. Once freed of these compounds, Rubisco can be activated by spontaneous carbamylation with CO_2 (89). Besides its carboxylation reaction, Rubisco also reacts with O_2, leading to photorespiration, an apparently wasteful reaction that lowers the efficiency of carbon incorporation to organic compounds. Oxygenic photosynthesis produces various radicals and active oxygen species with harmful effects on photosystem II (PSII). The excellent design of PSII gives protection to most of the protein components. Repair of PSII via turnover of the damaged protein subunits is a complex process involving (i) highly regulated reversible phosphorylation of several PSII core subunits, (ii) monomerization and migration of the PSII core from the grana to the stroma lamellae, (iii) partial disassembly of the PSII core monomer, (iv) highly specific proteolysis of the damaged proteins, and finally (v) a multi-step replacement of the damaged proteins with de novo synthesized copies followed by (vi) the reassembly, dimerization, and photoactivation of the PSII complexes (90). It has been reported that OEE2 (oxygen-evolving

enhancer protein 2-1) (23 kDa) and two other molecules (OEE1: oxygen-evolving enhancer protein 1-1 (33Kda) and OEE1-2; oxygen-evolving enhancer protein 1-2 (17 kDa) form an oxygen-evolving complex (OEC). They bind four Mn+2 ions that function in the splitting of H2O and maintain the environment for high rates of O2 evolution. With the aid of the oxygen-evolving complex, P680, a photosystem II (PSII)-reaction-center chlorophyll, oxidizes H2O to remove electrons; a total of two H2O molecules are split into four protons, four electrons, and one O2 molecule. Ballottari *et al*. (2007), demonstrated the ability of plants to acclimate into different environments and avoid photoinhibition by a faster activation rate for energy dissipation (qE), correlated to higher accumulation levels of a specific photosystem II subunit. In support of our speculation, the OEE2 decrease observed in all stress treated plants could be an index of their major sensibility to the freezing treatment in respect to plants that are previously acclimated at 4°C. Photosynthesis is greatly inhibited by low temperature in various crops. Our proteomic analysis showed that many photosynthetic proteins were partially degraded by chilling stress. Because the photosynthetic components are functionally linked, damage of any components may lead to the overall reduction of photosynthetic activity. In the spot 2309 (Fig. 27a and c) we found two proteins belonging to the same family: a chlorophyll a-b binding protein 2 and CP26. The absence of these proteins in A+R at T3 correlate well with the level of pigment analyzed (Fig.16, 17, 18). The PSII associated light-harvesting complexes (LHCs) bind chlorophylls and carotenoids that are involved in both the harvesting and transfer of energy to the reaction center, and the harmless dissipation of excitation energy in excess of photosynthetic capacity. Thus, the PSII LHCs are critical branch-points for energy partitioning during photosynthesis. The peripheral antenna consists of trimeric complexes composed of LHCII proteins, the major LHC of higher

plant antennae. In between the peripheral LHCII and the reaction center there are three minor LHCs referred to as CP24, CP26, and CP29.

The primary metabolisms, such as the metabolism of carbon and nitrogen, need to be modulated to establish a new homeostasis under freezing stress. For instance it has been reported that the overexpression of a glutamine synthetase (spot 2523, Fig. 27g) increase photorespiration and enhanced freezing stress tolerance (91). Photorespiration can provide a route to regulate ROS production rapidly during stress response. Moreover in spot 2523, we identified a phosphoglycerate kinase involved in the Calvin cycle and glycolysis (Fig. 27h); in fact, one of the mechanisms that can protect overwintering plants against photoinibition is an increase rate of photosynthetic carbon metabolism. Recent literature suggests a role of phosphoglycerate kinase in maintaining efficient carbon metabolism that makes the *Arabidopsis* genotype under examination an high frost tolerant one.

Defense proteins. The spot 3311 is present only in acclimated plant with and without freezing treatment, and contained a 20KDa chaperonin (Fig. 27n). The expression of this protein, which is responsible for the assembly of Rubisco, is enhanced remarkably under cold stress (92,93), suggesting that this double co-chaperonin, which occurs exclusively in plant chloroplasts, has an important role in refolding denatured Rubisco. In this case the level of protein and its relative transcript are in agreement. The special importance of small heat shock proteins (sHsps) in plants is suggested by unusual abundance and diversity. Six classes of sHsps have been identified in plants based on their intracellular localization and sequence relatedness. In addition to heat stress, plant sHsps are also produced under other stress

conditions and at certain developmental stages. Induction of sHsp gene expression and protein accumulation upon environmental stresses point to the hypothesis that these proteins play an important role in stress tolerance.

In spot 3518, we found F3E228 protein, a glycine-rich protein (Fig. 27q). Our transcript analysis showed that this mRNA is accumulating only during acclimation; we can speculate that acclimated plants might benefit from the catabolism of GRP proteins, as a consequence of stress, because their degradation provides glycine, an osmoprotective compounds (94).

Other proteins are also linked to cold adaptation: elongation factor Tu (spot 2527, Fig. 27l), (95, 96); putative pentatricoptide repeat containg protein (spot 2526, Fig. 27i), (97, 98); peptidly prolyl cis-trans isomerase CYP20-3 (spot 4315, Fig. 27u), (99); and a putative initiation factor 5A (spot 4316, Fig. 27v), (100). In fact *Arabidopsis* plants defective in CYP20-3 are hypersensitive to oxidative stress created by osmotic shock.

Energy metabolism was altered under cold stress as revealed by the altered expression of ATP synthase β chain (spot 3518, Fig. 27o), (101); in fact, this protein was degraded, which unavoidably resulted in decrease ATP production through photophosphorylation and thus affected the Calvin cycle.

Tab. 5. Protein identification by ESI-Quad TOF analysis

ESI

Spot (a)	Acc. No. (b)	Protein name	Organism	Gene reference (c)	Sequence Coverage (d)	Score (e)	Match (f)	Mw kDa Obs/Theo (g)		pI Obs/Theo (h)	
2309	P0CJ48	Chlorophyll a-b binding protein 2 chloroplastic	*Arabidopsis thaliana*	At1g29910	12%	174	4	26,5	28,3	5,00	5,29
	Q9T0L1	Putative uncharacterized protein	*Arabidopsis thaliana*	At4g13400	2,9%	67	2	26,5	34,6	5,00	4,99
	Q9XF89	Chlorophyll a-b binding protein CP26, chloroplastic	*Arabidopsis thaliana*	At4g10340	7,5%	107	2	26,5	30,1	5,00	6
2402	P10896	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	*Arabidopsis thaliana*	At2g39730	40,5%	1561	27	31,6	52,4	5,13	5,87
	P23321	Oxygen-evolving enhancer protein 1-1, chloroplastic	*Arabidopsis thaliana*	At5g66570	44%	783	19	31,6	35,4	5,13	5,55
2518	P10896-2	Isoform Short of Ribulose bisphosphate carboxylase/oxygenase activase chloroplastic	*Arabidopsis thaliana*	At5g66570	32,3%	1022	19	43,3	49,3	5,43	7,55
2523	Q9FWW5	T28K15.11 protein	*Arabidopsis thaliana*	At1g12150	2,7	42	2	46,7	62,8	5,11	5,37
	Q43127	Gllutamine synthetase, chloroplastic/mitochondria	*Arabidopsis thaliana*	At5g35630	7%	108	3	46,7	47,8	5,11	6,43
	P50318	Phosphoglycerate kinase, chloroplastic	*Arabidopsis thaliana*	At1g56190	4,2%	91	2	46,7	50,0	5,11	6,23
2526	Q9C7F1	Putative pentatricopeptide repeat-containing protein	*Arabidopsis thaliana*	At1g28020	1,8%	32	1	44,4	62,3	5,22	8,54
2527	P17745	Elongation factor Tu, chloroplastic	*Arabidopsis thaliana*	At4g20360	2,9%	58	2	42,9	51,9	5,19	5,84
	P10896	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	*Arabidopsis thaliana*	At2g39730	22,2%	638	14	42,9	52,3	5,19	5,87
3311	O65282	20 kDa chaperonin, chloroplastic	*Arabidopsis thaliana*	At5g20720	29,6%	316	7	24,5	26,8	5,45	8,86
3518	P19366	ATP synthase subunit beta, chloroplastic	*Arabidopsis thaliana*	AtCg00480	51,4%	1009	22	57,7	54,0	5,59	5,38
	O81016	ABC transporter G family member 32 (fragment)	*Arabidopsis thaliana*	At2g26910	1%	40	2	57,7	161,9	5,59	8,26
	Q9M7Y4	F3E22.8 protein	*Arabidopsis thaliana*	At3g06780	3,5%	31	2	57,7	21,7	5,59	6,82
	O03042	Ribulose bisphosphate carboxylase large chain	*Arabidopsis thaliana*	AtCg00490	21,3%	235	6	57,7	53.4	5,59	5,88
3603	P10896	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	*Arabidopsis thaliana*	At2g39730	43,2%	1020	20	60,4	52,6	5,45	5,87
4314	Q42029-2	Isoform 2 of Oxygen-evolving enhancer protein 2-1, chloroplastic	*Arabidopsis thaliana*	At1g06680	29,2%	244	6	21,0	23,8	5,76	7,71
4315	P34791	Peptidyl-prolyl cis-trans isomerase CYP20-3,	*Arabidopsis thaliana*	At3g62030	12,7%	149	3	19,4	28,5	5,70	8,83

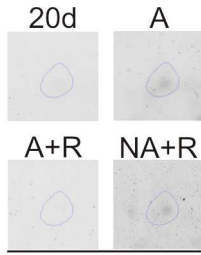|												
		chloroplastic										
4316	Q547G3	Putative initiation factor 5A	*Arabidopsis thaliana*	AT1G26630	20,8%	78	3	19,0	17,4	5,83	5,55
4513	P10896-2	Isoform Short of Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	*Arabidopsis thaliana*	At2g39730	15,2%	385	10	37,0	49,3	5,74	7,55
5615	P10896-2	Isoform Short of Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic	*Arabidopsis thaliana*	At2g39730	15,2%	292	8	62,2	49,3	6,18	7,55
5617	O03042	Ribulose bisphosphate carboxylase large chain	*Arabidopsis thaliana*	AtCg00490	28,4%	689	14	54,1	53,4	6,08	5,88
6610	O03042	Ribulose bisphosphate carboxylase large chain	*Arabidopsis thaliana*	AtCg00490	16,5%	301	8	57,6	53,4	6,43	5,88
6611	O03042	Ribulose bisphosphate carboxylase large chain	*Arabidopsis thaliana*	AtCg00490	39,7%	1008	31	52,4	53,4	6,32	5,88

Table 5. List of identified proteins by ESI-Quad TOF analysis and UniProtKB. (a) Spot number as indicate by PDQuest analysis; (b) UniProKB accession number; (c) AGI Gene reference code; (d) protein sequence coverage description; (e) Mascot score; (f) number of matched peptides; (g) gel-observed vs. theoretical molecular weights; (h) gel-observed vs. theoretical isoelectric points.

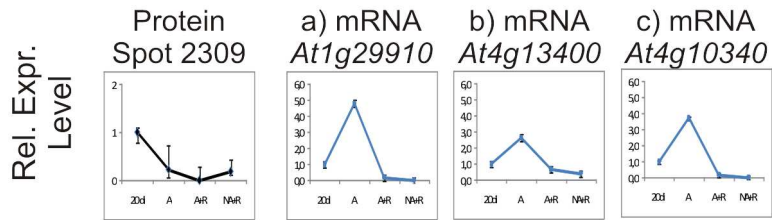**Tab. 6. Protein identification by MALDI-TOF MS analysis**

MALDI										
Spot (a)	**Acc. No. (b)**	**Protein name**	**Organism**	**Gene reference (c)**	**Sequence Coverage (d)**	**Score (e)**	**Match (f)**	**Mw kDa Obs/Theo (g)**		**pI Obs/Theo (h)**	
2307	Q42276	CAB binding protein,photosystem II (Fragment)	*Arabidopsis thaliana*	At1g06950.1	23,8%	63	4	26,1	10,85	5,12	6,1
3609	Q8LPR9	Protein TIC110, chloroplastic	*Arabidopsis thaliana*	At1g06950	22,3%	62	16	73,3	112,56	5,7	5,73

Table 6. List of identified proteins by MALDI-TOF peptide mass fingerprint analysis. (a) Spot number as indicate by PDQuest analysis; (b) UniProtKB accession number; (c) AGI Gene reference code; (d) observed vs. theoretical isoelectric points; (e) Mascot score; (f) percentage sequence coverage; (g) number of matched peptides; (h) biological process obtained after protein blast analysis.
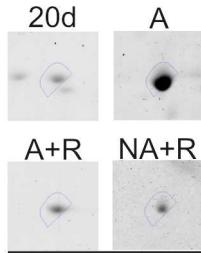
20d | A
A+R | NA+R

Spot 2309

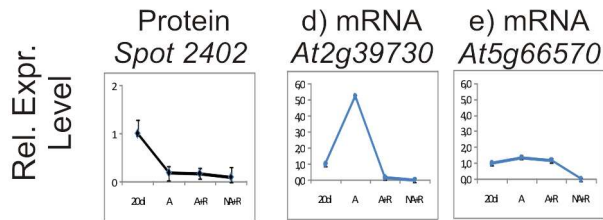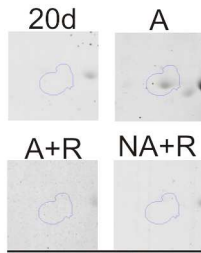Protein Spot 2309 | a) mRNA *At1g29910* | b) mRNA *At4g13400* | c) mRNA *At4g10340*

Refering to protein: a)Chlorophyll a-b binding protein 2, chloroplastic   b) Putative uncharacterized protein AT4g13400
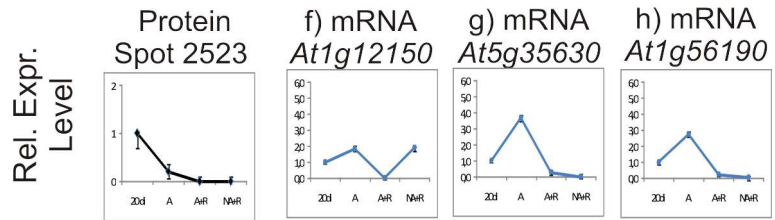c)Chlorophyll a-b binding protein Cp26 chloroplastic

20d | A
A+R | NA+R

Spot 2402

Protein *Spot 2402* | d) mRNA *At2g39730* | e) mRNA *At5g66570*

Refering to protein: d)Ribulose bisphosphate carboxylase/oxygenase activase chloroplastic
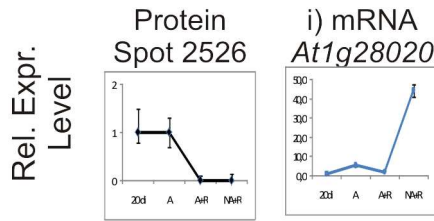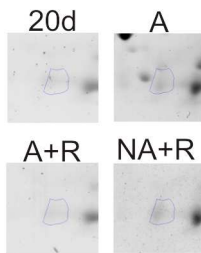e)Oxygen-evolving enhancer protein 1-1 chloroplastic

20d | A
A+R | NA+R

Spot 2523

Protein Spot 2523 | f) mRNA *At1g12150* | g) mRNA *At5g35630* | h) mRNA *At1g56190*

Refering to protein: f)T28K15.11 protein g)Glutamine synthetase chloroplastic
h)Phosphoglycerate kinase chloroplastic

20d | A
A+R | NA+R

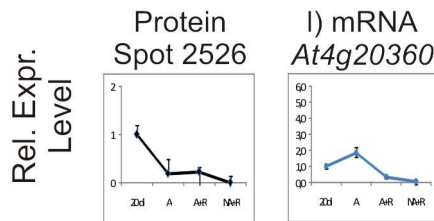Spot 2526

Protein Spot 2526 | i) mRNA *At1g28020*
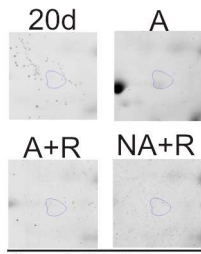
Refering to protein: i)Putative pentatricopeptide repeat-containing protein

20d | A
A+R | NA+R

Spot 2527

Protein Spot 2526 | l) mRNA *At4g20360*

Refering to protein: l)Elongation factor Tu chloroplastic

Rel. Expr. Level

20d A

A+R NA+R

Spot 3311

Protein Spot 3311

n) mRNA *At5g20720*
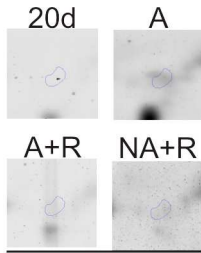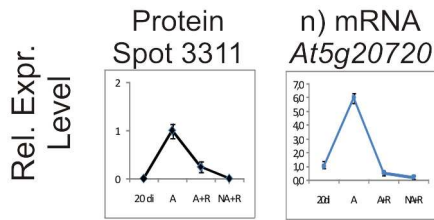
Refering to protein: n)20 kDa chaperonin chloroplastic

20d A

A+R NA+R

Spot 3518

Protein Spot 3518

o) mRNA *AtCg00480*

p) mRNA *At2g26910*

q) mRNA *At3g06780*

r) mRNA *AtCg00490*
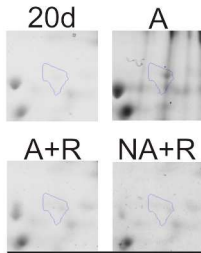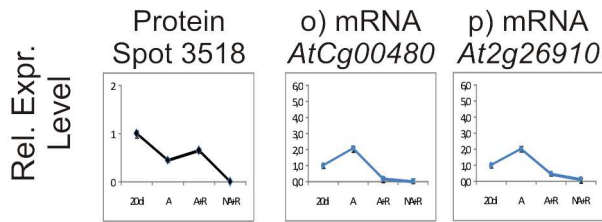
Refering to protein: o)ATP synthase subunit beta, chloroplastic p) ABC transporter G family member 32 q)F3E22.8 protein r)Ribulose bisphosphate carboxylase large chain

20d A

A+R NA+R

Spot 3609

Protein Spot 3609

s) mRNA *At1g06950*
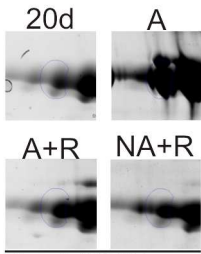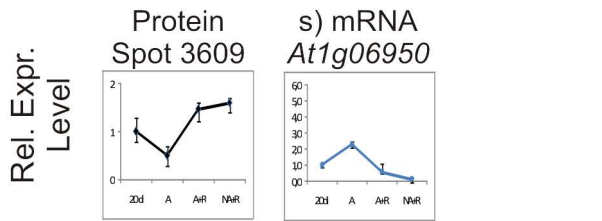
Refering to protein: s)At1g06940/F4H5_1

20d A

A+R NA+R

Spot 4314

Protein Spot 4314

t) mRNA *At1g06680*
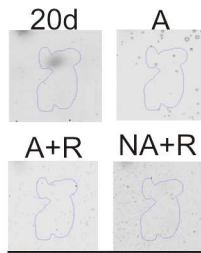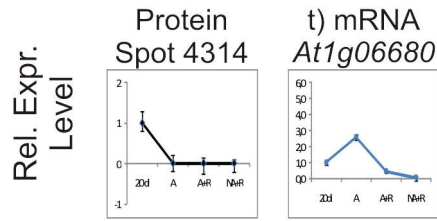
Refering to protein: t)Isoform 2 of Oxygen-evolving enhancer protein 2-1 chloroplastic
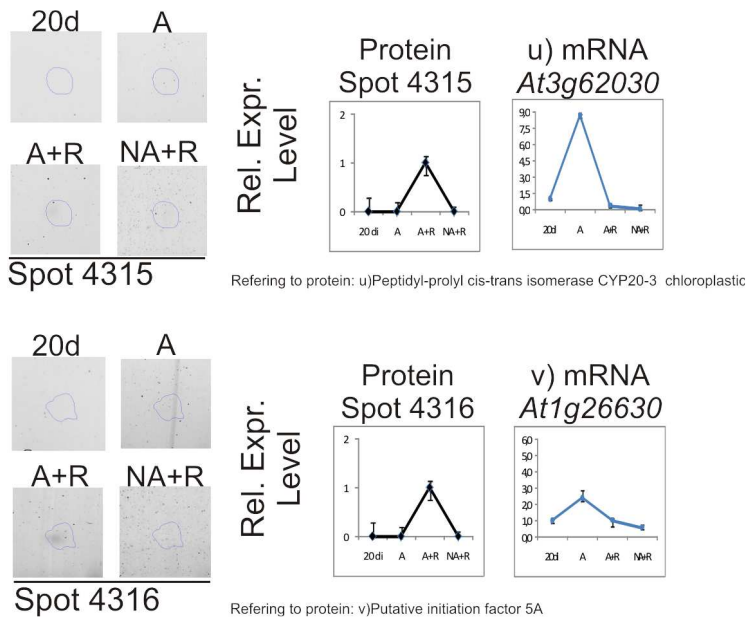
Figure 27. Comparison of protein and mRNA levels for proteins identified.
Zoom-in views of protein spots. A representative image is shown**.** Pattern of expression of each protein spots and corresponding mRNA. Protein expression levels data are the means of three replicates (± SD). mRNA expression was analysed by real time RT-PCR. Expression levels were calculated relative to that of 20 di = 1, in the case that the spot is absent (3311, 4315, 4316) in the control condition we used as reference A = 1. Data are the means of three replicates (± SD).

Chapter 4- Conclusion

Results concerning freezing tolerance in *Arabidopsis* differ slightly among the investigators. Differences in cold-acclimation conditions and in freezing treatment, including both light quantity and temperature, probably explain the differences reported. Beside, since plant cells are rich in many compounds (such as pigments, lipids, and polyphenols) that negatively affect protein extraction and disturb the first dimension of electrophoresis, methodological developments were necessary to find the best extraction procedure compatible with 2-DE gel analysis. Central goals in cold acclimation and freezing tolerance research include identifying involved proteins, determining how they are regulated and understanding their roles in plant life at low temperature. Most of the studies to date have been at genetic and transcriptomic levels. With the development of proteomic technologies these issues can be addressed on a more global scale. The obtained results demonstrate the capacity of proteomic approaches to analyze the cellular mechanisms at the level of proteins in order to understand the complexity of plant defense responses, despite current technical limitations.

4.1 *How to reach a new equilibrium under cold stress?*

Abiotic stress has an ability to alter the levels of a number of proteins. The most critical function of plant cell is to respond to stress by developing defense mechanisms. This defense is brought about by the alteration in the pattern of gene expression. This leads to modulation of certain metabolic and defensive pathways owing to altered gene expression under stress. We found a procedure that allows obtaining pure and intact chloroplasts from *Arabidopsis* leaves. In order to validate this result we employed a method for

the estimation of chlorophyll concentration and another one to validate chloroplast intactness. To date, this protocol is simple and rapid and useful in characterizing physiological properties of chloroplasts purified from the *Arabidopsis* model organism. This procedure allows analyzing the impact of *Arabidopsis* under stress conditions. The description of *A. thaliana* ecotype Columbia as a freezing-tolerant species was reported by various authors (102, 103, 104; 105; 106). We used ecotype Columbia-0 to study freezing-tolerance mechanisms in plants previously acclimated or not. It is well established that younger leaves exposed to a lower duration of frost may significantly increase freezing resistance in *Arabidopsis* (107).

Acclimated plants are better equipped than non-acclimated plants to withstand extreme environmental condition because of the more complex regulatory pathways triggered during the acclimative phase. In conclusion we can say that low temperature lead to a reduction of leaves growth, a modulation of photosynthesis visible at: Calvin cycle enzymes, thylakoid components and pigments content, and in change on stress-related proteins. In higher plants subjected to environmental conditions changes, such as low and freezing temperatures, photosynthetic tissues have to adjust their photosynthetic capacity. This involves adjusting the chloroplastic antennas, which are composed of pigments (mostly carotenoids) and proteins. When low temperatures reduce the rate of CO_2 fixation, thus lowering the overall rate of photosynthesis, light may exceed the amount that can be utilized in photosynthetic electron transport. If this excess of energy is not dissipated, the photosynthetic apparatus can be damaged, resulting in a decrease of photosystem II activity. This excess energy can be eliminated by thermal dissipation through a process known as non photochemical quenching and plants that can cold acclimate have more efficient mechanisms to do so. If the absorbed energy exceeds both the photochemical and non photochemical

quenching capacities, the result is irreversible photoinhibition or photodamage. Damages to the chloroplast structure disrupt its photosynthetic capacity. It has been suggested that photosynthesis could likely function as a sensor of this imbalance through the redox state of photosynthetic electron-transport components, resulting in the regulation of photochemical and metabolic processes in the chloroplast (35). Since light is essential for the development of maximal freezing tolerance, it appears likely that photosynthesis, and perhaps other processes taking place in the chloroplast, are crucial to cold acclimation and freezing tolerance. Moreover the ratio of Fv/Fm (variable fluorescence/maximum fluorescence) gives an estimate of the maximum quantum efficiency of PSII photochemistry, which has been widely used to detect stress-induced perturbation in the photosynthetic apparatus. Huner *et al.* (108, 109) reported that freezing tolerance of cereals was correlated with an increased capacity for photosynthesis upon cold acclimation. Thus, any factor that directly or indirectly affects photosynthesis could finally influence the freezing tolerance. A large suppression of Fv/Fm was observed after chilling treatment. This decrease was mostly reversible within a day of warming in growth chamber for A+R samples, but not for plants which were not acclimated (NA+R).

We can conclude that low temperature exacerbates an imbalance between the source of energy and the metabolic sink, thus adjustments of the photosynthetic apparatus to maintain the balance of energy flow during cold acclimation seems to be very important for cold tolerance. Moreover the electron transport rate of PSII (ETR) was higher in cold acclimated plants than in non-acclimated plants at low temperature and we can observe that acclimated plant recover completely the typical ETR levels of control plants.

Conclusion

The study was aimed at the identification of proteins involved in the response of *Arabidopsis thaliana* to freezing temperatures, by a proteomic approach focused on chloroplast. Results demonstrated that there were changes in protein expression in the total soluble cellular proteins isolated from 20 days-old *Arabidopsis* leaves after freezing stress, both in previously cold-acclimated plants and in non acclimated plants. Among the differentially expressed proteins, some were shared by all the condition of stress-treated plants while some others were peculiar of only one condition (for instance, 20kDa chaperonin). The results presented here indicate that both up-regulation and down-regulation of protein expression may be an important component of the adaptation to low temperature. These proteins encompassed a wide range of functions, including signalling, protein biogenesis, defence, and photosynthesis. It is possible that some of the identified proteins do not play direct roles in life at low temperature *per se* but instead play critical roles in enabling plants to adjust to quickly fluctuating environmental conditions, including protection against conditions that result in oxidative stress

Proteomics of chloroplasts and other plastid types has provided extensive protein inventories as well as information about PTMs, protein abundances, and protein interactions. Proteomics and MS technologies feeding into plastid proteome information now allow system-level analysis of chloroplast biology, including chloroplast development, signalling and interaction networks. We consider a high-quality plastid proteome atlas a milestone in the quest for biologically meaningful systems biology approaches. Together with parallel efforts for other organelles (e.g. mitochondria and peroxisomes), this will help to drive a better understanding of plant growth and development and help to realize the potential of plant systems biology.

5.2 Future perspectives

- *Could adaptation to altitude affect Arabidopsis thaliana stress tolerance?*

Low temperature defines a climatic range boundary for *Arabidopsis*, making temperature an interesting candidate for a habitat parameter that influences the genetic structure of local populations through natural selection. Such selection should be manifest by a relationship between freezing tolerance and the minimum habitat temperature during the growing season.

Using different ecotype of *Arabidopsis*, growing at various altitudes, we want to demonstrate that freezing tolerance of natural accessions correlates with habitat winter temperatures. Moreover, study how metabolite and transcript profiling vary during cold exposure could help us to correlate natural variation with the ability of *Arabidopsis* to cold acclimate (thanks to Marcel KUNTZ, for ecotypes collection).

References

1. J. Levitt, Responses of Plants to Environmental Stresses: Chilling, Freezing and High Temperatures Stresses. Second ed., *Academic press*, New York, 1980

2. Z. Ristic, E.N. Ashworth, Ultrastructural evidence that intracellular ice formation and possibly cavitation are the sources of freezing injury in supercooling wood tissue of *Cornus florida*. *Plant Physiol.,* 103 (1993) 753–761

3. I. Nishida, N. Murata, Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 47 (1996) 541–568

4. P.J. Christie, M.R. Alfenito, W. Walbot, Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta,* 194 (1994) 541–549

5. M.F. Thomashow, Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 50 (1999) 571–599

6. M. Griffith, H.C.H. McIntyre, The interrelationship of growth and frost tolerance in winter rye. *Physiol. Plant,* 87 (1993) 335-344

7. J.M. Anderson, W.S. Chow, Y.I. Park, The grand design of photosynthesis: Acclimation of the photosynthetic apparatus to environmental cues. *Photosynth Res.*, 46 (1995) 129–139

8. RG Walters, P Horton, Acclimation of *Arabidopsis thaliana* to the light environment: changes in composition of the photosynthetic apparatus. *Planta,* 195 (1994) 248–256

9. I. Ensminger, F. Busch, N.P.A. Huner, Photostasis and cold acclimation: sensing low temperature through photosynthesis. *Physiol. Plant,* 126 (2006) 28-44

10. N.P.A. Huner, G. O¨quist, V.M. Hurry, M. Krol, S. Falk, M. Griffith, Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. *Photosynth. Res.*, 37 (1993) 19-39

References

11. K.E. Wilson, A.G. Ivanov, G. Öquist, B. Grodzinski, F. Sarhan, N.P.A. Huner, Energy balance, organellar redox status, and acclimation to environmental stress. *Can J Bot.*, 84 (2006) 1355–1370

12. M.J. Hovenden, C.R. Warren, Photochemistry, energy dissipation and cold-hardening in *Eucalyptus nitens* and *E. pauciflora*. *J. Plant Physiol.*, 25 (1998) 581-589

13. G. Öquist, V.M. Hurry, N.P.A. Huner, Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. *Plant Physiol.*, 101 (1993) 245-250

14. F. Rizza, D. Pagani, A.M. Stanca, L. Cattivelli, Use of chlorophyll fluorescence to evaluate the cold acclimation and freezing tolerance of winter and spring oats. *Plant Breed*, 120 (2001) 389-396

15. P. Bertin, J. Bouharmont, J.M. Kinet, Somaclonal variation and improvement of chilling tolerance in rice: changes in chilling-induced chlorophyll fluorescence. *Crop Sci.*, 37 (1997) 1727-1735

16. W. Bilger, U. Schreiber, Energy dependent quenching of dark level chlorophyll fluorescence in intact leaves. *Photosynth. Res.*, 10 (1986) 303-308

17. W.L. Butler, Energy distribution in the photochemical apparatus of photosynthesis. *Annu. Rev. Plant Physiol.*, 29 (1978) 345-378

18. P.H. Lambrev, T. Tsonev, V. Velikova, K. Georgieva, M.D. Lambreva, I. Yordanov, L. Kovacs, G. Garab, Trapping of the quenched conformation associated with non-photochemical quenching of chlorophyll fluorescence at low temperature. *Photosynth Res.*, 94 (2007) 321–332

19. S. Schoner, G.H. Krause, Protective systems against active oxygen species in spinach: responses to cold acclimation in excess light. *Planta*, 180 (1990) 383–389

20. A. Matsui, J. Ishida, T. Morosawa, Y. Mochizuki, E. Kaminuma, T.A. Endo, M. Okamoto, E. Nambara, M. Nakajima, M. Kawashima, M. Satou, J.M. Kim, N. Kobayashi, T. Toyoda, K. Shinozaki, M. Seki, *Arabidopsis* transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array. *Plant Cell Physiol.*, 49 (2008) 1135–1149

References

21. G. Zeller, S.R. Henz, C.K. Widmer, T. Sachsenberg, G. Rätsch, D. Weigel, S. Laubinger, Stress-induced changes in the *Arabidopsis thaliana* transcriptome analyzed using whole-genome tiling arrays. *Plant J.*, 58 (2009) 1068–1082

22. B.H. Lee, D.A. Henderson, J.K. Zhu, The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell,* 17 (2005) 3155–3175

23. J.Z. Zhang, R.A. Creelman, J- K. Zhu, From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol.*, 135 (2004), 615–621

24. Z. Xin, J. Browse, Eskimo1 mutants of *Arabidopsis* are constitutively freezingtolerant, *Proc. Natl. Acad. Sci.*, USA 95 (1998) 7799–7804

25. L.A. Wanner, O. Junttila, Cold-induced freezing tolerance in *Arabidopsis.* *Plant Physiol.*, 120 (1999) 391–400

26. S.J. Gilmour, A.M. Sebolt, M.P. Salazar, J.D. Everard, M.F. Thomashow, Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiol.*, 124 (2000) 1854–1865

27. M. Smallwood, D.J. Bowles, Plants in a cold climate. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 357 (2002) 831–847

28. F. Llorente, R.M. Lopez-Cobollo, R. Catalá, J.M. Martinez-Zapater, J. Salinas, A novel cold-inducible gene from *Arabidopsis*, RCI3, encodes a peroxidase that constitutes a component for stress tolerance. *Plant J.,* 32 (2002) 13–24

29. M.J. Paul, C.H. Foyer, Sink regulation of photosynthesis. *J Exp Bot.,* 52 *(*2001*)* 1383–1400

30. G Papageorgiou, Govindjee, Chlorophyll a fluorescence. A signature of photosynthesis. Springer, Dordrecht, *The Netherlands*; (2004)

31. N.P.A Huner, M. Krol, J.P. Williams, E. Maissan, P. Low, D. Roberts, J.E. Thompson, Low temperature development induces a specific decrease in trans-D3-hexadecenoic acid content which influences LHCII organization. *Plant Physiol.*, 84 *(*1987*)* 12–18

32. M. Slot, C. Wirth, J. Schumacher, G.M.J. Mohren, O. Shibistova, J. Lloyd, I. Ensminger, Regeneration patterns in boreal Scots pine glades linked to cold-induced photoinhibition. *Tree Physiol.,* 25 *(*2005*)* 1139–1150

33. N.P.A Huner, G. Öquist, A. Melis, Photostasis in plants, green algae and cyanobacteria: the role of light harvesting antenna complexes. *In:* GreenBR, ParsonWW *(eds)* Advances in Photosynthesis and Respiration Light Harvesting Antennas in Photosynthesis. *Kluwer Academic Publishers*, Dordrecht, *(*2003*)* 401–421

34. M. Stitt, V. Hurry, A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. *Curr Opin Plant Biol,* 5 *(*2002*)* 199–206

35. I. Ensminger, F. Busch., N.P.A Huner, Photostasis and cold acclimation: sensing low temperature through photosynthesis. *Physiol Plantarum*, 126 (2006) 28-44

36. V. Chinnusamy., J. Zhu, J.K. Zhu, Gene regulation during cold acclimation in plants. *Physiol. Plant,* 126 (2006) 52-61

37. L. Xiong, H. Lee, M. Ishitani, Y. Tanaka, Repression of stress-responsive genes by FIERY2, a novel transcriptional regulator in *Arabidopsis*. Proc. *Natl. Acad. Sci. USA,* 99 (2002) 10899-10904

38. A. Abdrakhamanova, Q.Y. Wang, L .Khokhlova, P. Nick, Is microtubule disassembly a trigger for cold acclimation? *Plant Cell Physiol*, 44 (2003)676-686

39. K. Yamaguchi-Shinozaki, K. Shinozaki, Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.,* 57 (2006) 781-803

40. B.H. Lee, D.A. Henderson, J.K. Zhu, The *Arabidopsis* cold-responsive transcriptome and its regulation by ICE1. *Plant Cell,* 17 (2005) 3155-3175

41. F. Kaplan, J. Kopka, D.W. Haskell, W. Zhao, K.C. Schiller, N. Gatzke, D.Y. Sung, C.L. Guy, Exploring the temperature-stress metabolome of *Arabidopsis*. *Plant Physiol.,* 136 (2004) 4159-4168

42. J.T. Vogel, D.G. Zarka, H.A. Van Buskirk, S.G. Fowler, M.F. Thomashow, Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis. Plant J.,* 41 (2005) 195-211

43. Z. Xin, A. Mandaokar, J. Chen, R.L. Last, J. Browse, *Arabidopsis* ESK1 encodes a novel regulator of freezing tolerance. *Plant J.,* 49 (2007)786-799.

44. S.J. Gilmour, S.G. Fowler, M.F. Thomashow, *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol. Biol.*, 54 (2004)767-781

45. V. Chinnusamy, J. Zhu, J.K. Zhu. Cold stress regulation of gene expression in plants. *TRENDS in Plant Science,* 12 (10) (2007) 444-451

46. C. Benedict, M. Geisler, J. Trygg, N. Huner, V. Hurry, Consensus by democracy. Using meta-analyses of microarray and genomic data to model the cold acclimation signalling pathway in *Arabidopsis. Plant Physiol.,* 141 (2006) 1219-1232

47. Y. Guo, L. Xiong, M. Ishitani, J.K. Zhu, An *Arabidopsis* mutation in translation elongation factor 2 causes superinduction of CBF/DREB1 transcription factor genes but blocks the induction of their downstream targets under low temperatures. *Proc. Natl. Acad. Sci. USA,* 99 (2002) 7786-7791

48. Z. Gong, C.H. Dong, H. Lee, J. Zhu, L. Xiong, D. Gong, B. Stevenson, J.K Zhu, A DEAD box RNA helicase is essential form RNA export and important for development and stress responses in *Arabidopsis. Plant Cell,* 17 (2005) 256-267

49. G. Thorlby, N. Fourrier, G. Warren, The SENSITIVE TO FREEZING gene, required for freezing tolerance in *Arabidopsis thaliana*, encodes a beta-glucosidase. *Plant Cell,* 16 (2004) 2192-2203

50. J.Y. Kim, S.J. Park, B. Jang, C.H. Jung, S.J. Ahn, C.H. Goh, K. Cho, O. Han, H. Kang, Functional characterization of a glycine-rich RNA-binding protein in *Arabidopsis thaliana* under abiotic stress conditions. *The Plant Journal,* 50 (2007) 439-451

51. C.G. Burd, G. Dreyfuss, Conserved structures and diversity of functions of RNA-binding proteins. *Science,* 265 (1994) 615-621

52. Z.J. Lorkovic´, A. Barta, Genomic analysis: RNA recognition motif (RRM) and K homology (KH) domain RNA-binding proteins from the flowering plant *Arabidopsis thaliana*. *Nucleic Acids Res.*, 30 (2002) 623-635

53. R. Hill, Oxygen evolution by isolated chloroplasts. *Nature,* 139 S (1937) 881-882

54. R. Hill, Oxygen produced by isolated chloroplasts. *Proc. R. Soc. London Ser. B,* 127 (1939) 192-210

55. D.I. Arnon, M.B. Allen and F.R. Whatley, (1954) Photosynthesis by isolated chloroplasts. *Nature,* 174 (1954) 394-396

56. D.A. Walker, Improved rates of carbondioxide fixation by illuminated chloroplasts. *Biochem. J.,* 92 (1964) 22C-23C

57. D.A. Walker, Chloroplast (and grana): Aqueous (including high carbon fixation ability). *Methods Enzymol.* 23 (1971) 211-220

58. R.G. Jensen and J.A. Bassham, Photosynthesis by isolated chloroplasts. *Proc. Natl. Acad.Sci. U.S.A.* 56 (1966) 1095-1101

59. U. Heber, K.A. Santarius, Direct and indirect transfer of ATP and ADP across chloroplast envelope. *Z. Naturforsch.*, Part B 25 (1970) 718-728.

60. H.W. Heldt, F. Sauer, Inner membrane of chloroplast envelope as site of specific metabolite transport. *Biochim. Biophys. Acta.*, 234 (1971) 83-91

61. H. Pertoft, Fractionation of cells and subcellular particleswith Percoll. *J. Biochem. Biophys. Methods,* 44 (2000) 1-30

62. C.R. Somerville, S.C. Somerville, W.L. Ogren, Isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*. *Plant Sci. Lett.*, 21 (1981) 89-96

63. L. Kunst, Preparation of physiologically active chloroplasts from *Arabidopsis*. In *Methods in Molecular Biology*, 82 (1998): *Arabidopsis* Protocols (J. M. Martinez-Zapater, and J.Salinas, eds.) 43-48. *Humana Press Inc*, Totowa, N.J.

References

64. M. Ferro, D. Salvi, , S. Brugi`ere, S. Miras, S. Kowalski, M. Louwagie, J. Garin, J. Joyard and N. Rolland, Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. *Mol. Cell Proteomics,* 2 (2003) 325-345

65. R. Aebersold and M. Mann, Mass spectrometry-based proteomics, *Nature,* 422 (2003) 198–207

66. W. Weckwerth, S. Baginsky, K. vanWijk, JL. Heazlewood, H. Millar, The multinational arabidopsis steering subcommittee for proteomics assembles the largest proteome database resource for plant systems biology. *J Proteome Res*, 7 (2008) 4209–10

67. S. Baginsky, Plant proteomics: concepts, applications, and novel strategies for data interpretation. *Mass Spectrom Rev.*, 28 (2009) 93–120

68. KJ. van Wijk, Plastid proteomics. *Plant Physiol Biochem*, 42 (2004) 963–77.

69. T. Kleffmann, D. Russenberger, A. von Zychlinski, W. Christopher, K. Sjolander, W. Gruissem, The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. *Curr Biol*, 14 (2004) 354–62

70. O. Emanuelsson, S. Brunak, G. von Heijne, H. Nielsen, Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc*, 2 (2007) 953–71

71. B. Zybailov, H. Rutschow, G. Friso, A. Rudella, O. Emanuelsson, Q. Sun, Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS ONE,* 3 (2008) 1994

72. M. Ferro, S. Brugiere, D. Salvi, D. Seigneurin-Berny, M. Court, L. Moyet, C. Ramus, S. Miras, M. Mellal, S. Le Gall, S. Kieffer-Jaquinoid, C. Bruley, J. Grarin, J. Joyard, C. Masselon, N. Rolland, AT_CHLORO: A comprehensive chloroplast proteome database with subplastidial localization and curated information. *Molecular & Cellular Proteomics* 9 (2010) 1063-1084

73. A.M. Gilmore, H.Y. Yamamoto, Zeaxanthin formation and energy-dependent fluorescence quenching in pea chloroplasts under artificially mediated linear and cyclic electron transport. *Plant Physiol.*, 96 (1991) 635-643

74. D.V. Vavilin, *Journal of Photochemistry and photobiology,* 42 (1998) 233-239

75. B.R. Oakley, D.R. Kirsch, N.R. Morris, A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* 105 (1980) 361-363

76. B. Granvogl, M. Plöscher, L. Eichacker, Sample preparation by in-gel digestion for mass spectrometry-based proteomics. *Anal. Bioanal. Chem.* 389(4) (2007) 991-1002

77.D. Salvi, L. Moyet, D. Seigneurin-Berny, M. Ferro, J. Joyard, N. Rolland, Preparation of envelope membrane fractions from *Arabidopsis* chloroplasts for proteomic analysis and other studies. *Method in molecular Biology,* 775(3) (2011) 189-206

78. D. Walker, The use of the oxygen electrode and fluorescence probes in simple measurements of photosynthesis. *Oxygraphics Ltd*, University of Sheffield, South Yorkshire, UK. (1990)

79. G.H. Krause, E. Weis, Chlorophyll fluorescence and photosynthesis: the basics. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 42 (1991) 313-349

80. G.R. Gray, B.J. Hope, X.Q. Qin, B.G. Taylor, C.L. Whitehead, The characterization of photoinhibition and recovery during cold acclimation in *Arabidopsis thaliana* using chlorophyll fluorescence imaging. *Plant Physiol.*, 119 (2003) 365-375

81. N.R. Baker, E. Rosenqvist, Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *J. Exp. Bot.,* 55 (2004) 1607-1621

82. M. Król, A.G. Ivanov, S. Jansson, K. Kloppstech, N.P.A. Huner, Greening under high light or cold temperature affects the level of xanthophyll-cycle pigments, early light-inducible proteins, and light-harvesting polypeptides in wild-type barley and the Chlorina f2 mutant. *Plant Physiol.*, 120 (1999) 193–203

83. K.E. Wilson, A.G. Ivanov, G. Öquist, B. Grodzinski, F. Sarhan, N.P.A. Huner, Energy balance, organellar redox status, and acclimation to environmental stress. *Can J Bot,* 84 *(*2006*)* 1355–1370

84. M.P. Johnson, M. Havaux, C. Triantaphylidès, B. Ksas, A.A. Pascal, B. Robert, P.A. Davison, A.V. Ruban, P. Horton, Elevated zeaxanthin bound to oligomeric LHCII enhances the resistance of *Arabidopsis* to photo-oxidative

stress by a lipid protective, anti-oxidant mechanism. *J Biol Chem,* 282 *(*2007*)* 22605–22618

85. A.M. Gilmore, M.C. Ball, Protection and storage of chlorophyll in overwintering evergreens. *Proc. Natl Acad. Sci. U S A,* 97 *(*2000) 11098-11101

86. M. L. Pérez-Bueno, P. Horton, The role of lutein in the acclimation of higher plant chloroplast membranes to suboptimal conditions, *Physiologia Plantarum,* 134 (2008) 227-236

87. A.R. Portis, Regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.,* 43 (1992) 415-437

88. M. E. Salvucci, W. L. Ogren, The mechanism of Rubisco activase: insights from studies of the properties and structure of the enzyme. *Photosynth. Res.,* 47(1996) 1-11

89. G.H. Lorimer, H.M. Miziorko, Carbamate formation on the epsilon-amino group of a lysyl residue as the basis for the activation of ribulosebisphosphate carboxylase by CO_2 and Mg^{2+}. *Biochemistry,* 19(23) (1980) 5321-5328

90. E.M. Aro, M. Suorsa, A. Rokka, Y. Allahverdiyeva, V. Paakkarinen, A. Saleem, N. Battchikova, E. Rintamaki, Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes. *Journal of Experimental Botany,* 56(411) (2004) 347-356

91. H. Hoshida, Y. Tanaka, T. Hibino, Y. Hayashi, A. Tanaka, T. Takebe, Enhanced tolerance to salt stress in transgenic rice that overexpresses chloroplast glutamine synthetase. *Plant Mol. Biol,* 43 (2000) 103-111

92. S. Cui, F. Huang, X. Ma, Y. Cheng, J. Liu, A proteomic analysis of cold stress responses in rice seedlings. *Proteomics,* 5 (2005) 3162-3172

93. A.K. Neilson, C. G. Gammulla, M. Mirzaei, N. Imin, P. A. Haynes, Proteomic analysis of temperature stress in plant. *Proteomics,* 10 (2010) 828-845

94. T. Nomata, Y. Kabeya, NV. Sato, Cloning and characterization of glycine-rich RNA-binding protein cDNAs in the moss *Physcomitrella patens. Plant Cell Physiology,* 45 (2004) 48–56

95. G. Cacace, M. F. Mazzero, A. Sorrentino, V. Spada, A. Malorni, R. A. Siciliano, Proteomics for the elucidation of cold adaptation mechanisms in *Listeria monocytogenes. J. of Proteomics*, 72 (2010) 2021-2030

96. L. Xiayan, S. R. Rodermel, F. Yu, A var2 leaf variegation suppressor locus, SUPPRESSOR OF VARIEGATION3, encodes a putative chloroplast elongation factor that is important for chloroplast development in the cold. *Plant Biology*, 10 (2010) 287

97. E. Kotera, M. Tasaka, T. Shikanai, A pentatricopeptide repeat proteins essential for RNA editing in chloroplasts. *Nature*, 433 (2005) 326-330

98. D. Saha, A.M. Prasad, R. Srinivasan, Pentatricopeptide repeat proteins and their emerging roles in plants stress response. *Plant Physiology and Biochemistry*, 45(8) (2007) 521-534

99. J. R. Dominguez Solis, Z. He, A. Lima, J. Ting, B.B. Buchanan, S. Luan, A cyclophilin links redox and light signals to cysteine biosynthesis and stress responses in chloroplasts. *National Acad Sciences*, 105(42)(2008) 16386-391

100. H. Feng, Q. Chen, J. Feng, J. Zhang, X. Yang, J. Zuo, Functional characterization of *Arabidopsis* initiation factors 5A-2 that play a crucial role in plant growth and development by regulating cell division, growth and death. *Plant Physiology*, 144 (2007) 1531-1545

101. A. Bocian, A. Kosmala, M. Rapacz, B. Jurczyk, L. Marczak, Z. Zwierzykowski, Differences in leaf proteome response to cold acclimation between *Lolium perenne* plants with distinct levels of frost tolerance. *J. of Plant Physiol*, 168 (11) (2011) 1271-1279

102. M.F. Thomashow, Molecular genetics of cold acclimation in higher plants. In Scandalios J.G. (Ed.). Advances in genetics, genomic responses to environmental stress. *Academic Press, New York* 28 (1990) 99-131

103. M.F. Thomashow. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50 (1999) 571-599

104. V. Lang, E. Mantyla, B. Welin, B. Sundberg, T. Palva, Alteration in water status, endogenous abscisic acid content, and expression of the *rab18* gene during the development of freezing tolerance in *Arabidopsis thaliana*. *Plant Physiology* 104 (1994) 1341-1349

References

105. G.J. Warren, R. McKown, A. Marin, R. Teutonico, Isolation of mutants affecting the development of freezing tolerance in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiology* 111 (1996) 1011-1019

106. S.J. Gilmour, D.G. Zarka, E.J. Stockinger, M.P. Salazar, J.M. Houghton, M.F. Thomashow, Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant J.* 16 (1998) 433-442

107. L.A. Wanner and O. Junttila, Cold induced freezing tolerance in Arabidopsis. *Plant Physiology* 120 (1999) 391-399

108. N.P.A. Huner, G. Oquist, V.M. Hurry, M. Krol, S. Falk, M. Griffith, Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. *Photosynth. Res.* 37 (1993) 19-39

109. N.P.A. Huner, G. Oquist, F. Sarhan, Energy balance and acclimation to light and cold. *Trends in Plant Science* 3 (1998) 224-230

Acknowledgements

…at the end of a path it must be stopped to reflect.

For this reason I take this opportunity to thank all the people that, during these years, have believed in me, in my abilities and my work.

Prof. Amedeo Alpi who encouraged me and has always supported my ideas.

Prof. Marcel Kuntz that in a short time managed to teach me so much and he has never refused a sincere help.

Dr. Matteo Ballottari who addressed me with great patience toward new assumptions and analyzes that have enriched my work.

My colleagues who have made the work pleasant lightening with laughter, advice and collaboration. Among all Federico, not just a colleague, but friend.

My old friends who, although not belonging to the research world, have always gone along with my craziness.

And of course my family without which nothing would be possible.

Valentina