Low temperature acclimation in plants
alterations in photosynthetic carbon metabolism

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Abstract:
Although low temperature plays an important role in determining agricultural yield, little is known about the effect on the underlying biochemical and physiological processes that influence plant growth. Photosynthesis and respiration are central to plant growth and both processes are heavily affected by temperature. However, many plants have the ability to cope with low temperature and resume growth by cold acclimating.

We have shown that enhancement of carbon fixation, an increased flux of carbon into sucrose and the recovery of diurnal export is crucial for the recovery of functional carbon metabolism at low temperature in Arabidopsis thaliana. The recovery of efflux is governed by increased expression of sucrose transporters along with changes in vascularisation. We also demonstrate the importance of controlling the flux of metabolites between the chloroplast and the cytosol by regulating the expression of AtTPT.

We further investigated the difference in response between leaves developed at low temperature but originating from warm grown Arabidopsis and leaves from plants grown from seed at low temperature. We were able to distinguish factors that respond specifically to low temperature from those that are connected to the actual stress. Substantial difference could be seen in the different metabolomes. One conclusion drawn is that the increase in sucrose reported at low temperature is an essential feature for life in the cold.

In an extended study we were able to transfer some of the key factors of cold acclimation in Arabidopsis to other species. The study included forbs, grasses and evergreen trees/shrubs showed that there are striking similarities in the extent and biochemical changes that underpin acclimation among the different functional groups.

Low temperature does not only influence growth of the leaves, perennial organs such as the corm of the ornamental plant Crocus vernus is also affected. However in these plants low temperature has a positive effect on the final size of the corm. We were able to show that this enhanced growth was an affect of increased cell size and thus increased sink capacity, which ultimately delays leaf senescence.

Key words: cold acclimation, carbon metabolism, photosynthesis, respiration, carbon storage

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Preface

Farmers around the world the constantly struggle against the environment and they have been fighting a winning battle, succeeding in increasing food production enough to keep up with the accelerating population increase. On the world market there is still a general surplus; by 1990 the production was large enough to, in theory, provide every human with nearly 4000 calories per day. In spite of this hunger and malnutrition is common and increasing in many countries. If the population increase continues as predicted, to approximately 9 billion by year 2050, the world food production needs to increase by 70% to keep up with the levels of 1990. This increase has to be achieved in a time when the world faces the prospect of a change in the global climate. Despite technical advances such as improved crop varieties and irrigation systems, weather and climate are still key factors affecting agricultural productivity.

Changes in the timing and duration of the growing seasons, anticipated with the global warming, is believed to increase the vulnerability to freezing damage from early or late season frost, as reported in the tundra (Molgaard and Christensen, 1997) and temperate forests (Norby et al., 2003). The prediction of plant responses are complicated by the fact that elevated CO₂ can lower the freezing tolerance, making even hardy plants more sensitive to frost damage at warmer temperatures (Loveys et al., 2006).

Boyer (1982) concluded that most crops, on average, only achieve around 20% of their inherent yield potential. The reduction is to a large extent an effect of abiotic stresses, thus by improving the abiotic stress tolerance of crop species a substantial increases in yield could be achieved. The processes underpinning the increase in freezing tolerance has received considerable attention from the scientific community, however, despite this, little progress has been made in improving freezing tolerance using traditional breeding strategies.

This thesis aims to increase the knowledge about how plants cope with low temperatures and how they go about increasing the freezing tolerance. The research described focuses on the responses of primary carbon metabolism, ultimately providing more information on how we potentially could increase the inherent freezing tolerance using more modern and specific tools.
Introduction

The capacity to sense and respond to changes in temperature varies greatly between species. Many economically important crops such as cotton, soybean, maize and rice are chilling sensitive and unable to survive freezing temperatures (Larcher, 1995), while others like Arabidopsis and crops like winter cereals, spinach and cabbage, are able cope with temperatures below freezing. Plants can, in general, be classified into three broad categories based on their response to decreasing temperatures. The first category is the chilling sensitive plants, e.g. rice and maize, which are irreversible damaged by temperatures below 10-15°C (Levitt, 1972). Plants that are chilling resistant show no dysfunction at temperatures above zero but are unable to develop freezing resistance (Sakai and Larcher, 1987). The final class, the freezing tolerant plants, include plants that have an inherent ability to develop freezing tolerance (Levitt, 1972; Sakai and Larcher, 1987). These plants are able to increase their tolerance by a process known as cold acclimation. Cold acclimation is induced by exposure to a period of low non-freezing temperatures that bring about genetic, morphological, and physiological changes, which results in the development of cold hardiness and the acquisition of freezing tolerance.

1. Consequences of low temperature stress

In the following sections I will give a broad overview of some of the effects and possible damages that occur when plants are subjected to low temperatures.

1.1. Ice formation, dehydration and its effects on membranes

Low temperature injury (i.e. chilling and freezing) can occur in all plants, but the mechanisms and types of damage vary considerably. Freezing injury is connected to the formation of ice crystals that occur once ice nucleation can no longer be avoided (Burke et al., 1976). The formation of ice is heavily dependent on the presence of ice nucleators, organic or inorganic substance that catalyse ice formation (Zachariassen and Kristiansen, 2000), and the cooling rate. It can to a certain extent, be prevented by the presence of solutes and by supercooling, allowing the cell fluid to be cooled down to temperature below freezing without ice nucleation. If the cooling rate is slow ice initiation occurs in the extracellular water, due to its lower solute concentration and higher levels of ice-nucleating agents. Without ice nucleators water remains in a supercooled state above -38°C, the temperature at which water self nucleates (Thomashow, 1998). In cold tolerant plants ice formation appear controlled, it starts at the outer surface of the cell wall and spreads through the extracellular areas (Pearce and Ashworth, 1992), suggesting that ice nucleators are produced at specific sites (Brush et al., 1994). Potent ice nucleating agents have been found in the extracellular fluid of species ranging from trees such as Prunus (Gross et al., 1988), Citrus (Constantinidou and Menkissoglu, 1992) to annual plants like winter rye (Secale cereale) (Brush et al., 1994). As long as the plasma membrane remains intact the ice is confined to the
outside of the cell (Levitt, 1980). Protrusion of ice into the cell is lethal, due to mechanical disruptions of the cell (Burke et al., 1976). The formation and containment is further influenced by antifreeze or thermal hysteresis proteins (Griffith et al., 1992; Urrutia et al., 1992; Griffith et al., 1997; Pihakaski-Maunsbach et al., 2001). These proteins are able to decrease the temperature at which ice is formed in a solution without affecting the melting point of the solution. That there is no modification of the melting point implies that once bound to the ice nuclei they may inhibit the growth of the ice crystal even when in contact with supercooled water (Zachariassen and Kristiansen, 2000). In addition they affect the shape of the ice crystal and help inhibit recrystallisation (i.e. they inhibit small ice crystals from joining producing larger ice crystals).

The formation of intercellular ice is also the major reason behind cellular dehydration at low temperature. The ice crystals decrease the water potential outside the cell and osmosis force water out, dehydrating the cell (Levitt, 1980; Webb and Steponkus, 1993). As water is removed, the solute concentration increase and the likelihood of freezing is reduced. However, with continued growth of the ice, the cells become more desiccated, with profound effects on the cellular membranes, with the plasma membrane as the primary site of injury (Steponkus, 1984), as well as causing denaturation and precipitation of protein and molecules (Guy, 1990; Thomashow, 1998).

Three types of freeze-induced membrane lesions have been characterized, depending on the stage of cold acclimation and the extent of freeze-induced dehydration (Steponkus, Uemura & Webb, 1993; (Uemura et al., 1995). In non-acclimated plants the dehydration give rise to two different lesions associated with the plasma membrane. During freezing, osmotic contractions results in endocytotic vesiculation beneath the plasma membrane. Approximately 40% of the membrane surface area was lost this way in protoplasts of rye (Secale cereale) subjected to freezing (Dowgert and Steponkus, 1984), the reduction in area is irreversible and as a consequence the cell lyses when expansion occur during thawing. This common feature of non acclimated cells of rye, Arabidopsis and oat is referred to as expansion-induced lysis (EIL) (Steponkus et al., 1988; Uemura and Steponkus, 1989; Webb et al., 1994), and is associated with temperatures ranging from -2 to -4°C. When the temperature drops further, to below -5°C, and more severe dehydration occur, the injury is manifested as a complete loss of osmotic responsiveness (LOR) during thawing. In non-acclimated tissue this is due to a phase transition of the phospholipids within the membrane from a lamellar (bilayer) to a hexagonal II (HII) phase when the water content of the cell drops to approximately 20%. During the HII-phase transition the phospholipids form long cylinders with the polar head groups orientated into the aqueous core rendering the membrane unable to expand (Gordon-Kamm and Steponkus, 1984; Uemura and Steponkus, 1994; Uemura et al., 1995).
1.2. Photosynthetic carbon metabolism

Photosynthesis is a highly regulated process transforming light energy into usable chemical energy in the form of ATP and NADPH for the assimilation of carbon and other essential nutrients. Proper function relies on a balance between the energy absorbed and trapped and the energy consumed by the metabolic reactions downstream. Low temperature has only a minor effect on the primary reactions of photosynthesis, catalysed by photosystem I and II, trapping the light energy and converting it into redox potential energy. A stronger effect can be seen on electron transport, increasing the viscosity of the thylakoid membrane, in addition to decreasing the rates of the enzymatic reactions of downstream metabolism (Huner et al., 1998). The balance between energy input through photochemistry and energy utilization through metabolism is called photostasis (Öquist and Huner, 2003) and since the temperature effects are skewed an imbalance is created. The shift in photostasis is further affected by the cessation in growth caused by low temperature inhibition of water and nutrient uptake. This disproportion may induce high excitation pressure and lead to photo-inhibition of photosynthesis (Melis, 1999). The primary metabolic sink, consuming the chemical energy is represented by the reduction of CO$_2$ to triose phosphate and the continuous regeneration of ribulose 1,5 bisphosphate (RuBP) in the Calvin cycle within the chloroplast. However, the assimilation of N and S also constitute sinks for photosynthetically generated redox equivalents and chemical potential energy (Paul and Foyer, 2001) and the effects of low temperature on these reactions are largely unknown. Optimal rates of photosynthesis are also dependent on a balance in carbon flow between the chloroplast and the cytosol. The most important pathway for end product synthesis in the cytosol is sucrose biosynthesis. If the rate of sucrose biosynthesis is running too fast it will inhibit photosynthesis by withdrawing too many intermediates from the Calvin cycle (Stitt, 1986; Stitt et al., 1987) and if it is too slow by the sequestering of inorganic phosphor (Pi) in phosphorylated intermediates (Stitt et al., 1987; Stitt 1986). The coordinated regulation of the key-enzymes of sucrose biosynthesis fructose-1,6-bisphosphatase (cFBPase) and sucrose phosphate synthase (SPS) are necessary for adjusting the synthesis to the supply of carbon.

Low temperature stress is known to cause a over-proportional repression of sucrose synthesis, the key regulatory enzymes together with UDP-glucose pyrophosphorylase are particularly sensitive (Stitt and Grosse, 1988). cFBPase converts fructose-1,6-bisphosphate to fructose-6-phosphate (F$_6$P), and is inhibited by AMP and by the signal molecule fructose-2,6-bisphosphate (F$_2$,6BP), which increase with decreasing levels of PGA and increasing levels of Pi. SPS situated downstream converts UDP-glucose and F$_6$P to sucrose-6-phosphate (Stitt et al., 1987) and is regulated both allosterically by glucose-6-phosphate (G$_6$P) and Pi, and by phosphorylation (Huber and Huber, 1996). Phosphorylation of SPS results in a reduction in enzyme activity due to a decrease in the affinity for F$_6$P and the allosteric activator G$_6$P (Fig. 3). The accumulation of soluble carbohydrates seen at low temperature cause inactivation of SPS by protein phosphorylation (Guy et al.,
1992; Huber and Huber, 1992; Weiner et al., 1992) and the associated accumulation of fructose-6-phosphate (F6P) triggers the synthesis of fructose-2,6-bisphosphate, (Stitt, 1990). This inhibition of end product synthesis results in a build up of phosphorylated intermediates within hours after exposure to low temperature (Labate and Leegood, 1988), decreasing the Pi cycling between the cytosol and the chloroplast. This depletion of the stromal pool of Pi restricts the CF1-ATPase activity (Furbank et al., 1987; Pammenter et al., 1993), hindering the synthesis of ATP needed for the regeneration of RuBP, thereby decreasing the rates of electron transport and contributing to down-regulation of photosynthesis (Labate and Leegood, 1988; Foyer et al., 1990).

The accumulation of soluble carbohydrates further affects the photosynthetic capacity by repressing the expression of a number of genes encoding proteins important for photosynthesis. The addition of glucose to Chenopodium cell cultures (Krapp et al., 1993) as well as cold girdling of spinach (Krapp and Stitt, 1995) caused a rapid decrease in mRNA levels of the small subunit of Rubisco, of chlorophyll a/b binding proteins and of the δ-subunit of the thylakoid ATPase. The change in gene expression resulted in a change in protein amount, 4-5 days after cold girdling for the amount of Rubisco had been reduced in half in leaves of tobacco, potato and spinach (Krapp et al., 1993; Krapp and Stitt, 1995). These alterations was confirmed in Arabidopsis plants shifted to 5°C (Strand et al., 1997).

1.3. Respiration
Respiration is indispensable for the release of the metabolic energy bound by photosynthesis. Respiration is also vital as a source of carbon skeletons exported to the cytosol for growth and cellular maintenance (Fernie et al., 2004). Moreover, mitochondrial respiration is central for the continuance of photosynthetic activity, mostly due to the high energy requirement of sucrose biosynthesis (Kršmer, 1995). Respiration involves oxidation of highly reduced carbohydrates through glycolysis in the cytosol and the tricarboxylic acid (TCA) cycle within the matrix of the mitochondria, releasing CO₂ and reducing equivalents (NAD(P)H and FADH₂). The NAD(P)H and FADH₂ generated transfers their electrons to O₂ via the mitochondrial electron transport chain, resulting in the consumption of oxygen and the release of ATP (Siedow and Day, 2000). Plant respiration is remarkably flexible with parallel glycolytic pathways in both the cytosol and plastid, alternative enzymes within glycolysis and the TCA cycle and the presence of both phosphorylating and non-ATP producing pathways of the electron transport chain (Plaxton, 1996; Siedow and Day, 2000; Rasmusson et al., 2004; Plaxton and Podesta, 2006). These features are suggested to be crucial aids for the acclimation to different stresses (Møller, 2001; Podesta, 2004; McDonald and Vanlerberghe, 2006; Plaxton, 2006).

Ultimately the equilibrium between photosynthesis and respiration is of great importance for the productivity and general function of the plant. Between 30 and
80% of the photosynthetically fixed carbon may be respired in the same day depending on species and growth conditions (Atkin et al., 1996; Tjoelker et al., 1999; Amthor, 2000; Loveys et al., 2002; Poorter and Navas, 2003). Respiration is known to be temperature sensitive (Wager, 1941; James, 1953; Forward, 1960). It is often assumed that the relationship between respiration and temperature is exponential with a constant $Q_{10}$, i.e. proportional changes in respiration with a 10°C increase in temperature, typically around 2. However, it has been recognised that $Q_{10}$ is not constant or near 2, except within a limited temperature range (Wager, 1941; James, 1953; Forward, 1960). Instead there is growing evidence that the thermal response varies among species and that it is dynamic, varying with the metabolic status of the plants, as well as acclimating to changes in temperature but also drought and light (Collier, 1996; Bryla et al., 1997; Atkin et al., 2000; Bryla et al., 2001; Griffin et al., 2002). Changes in respiration as a function of temperature can be regulated by the supply of adenylates and substrates, and by the capacity of the enzymes of respiration (Atkin et al., 2000). There is evidence that respiration is capacity limited at low temperature. At moderate temperatures (25°C) the addition of respiratory uncouplers and glucose is able to enhance the $O_2$ uptake in intact roots of two species of Plantago, while it failed to trigger any response at low temperature (Covey-Crump et al., 2002). Furthermore, the addition ADP do not affect the substrate saturated $O_2$ uptake in cold treated soybean cotyledons (Atkin et al., 2002). Finally, the fact that glycolytic substrates such as soluble sugars accumulates at low temperature suggests that respiratory flux is more likely to be controlled by the catalytic activity of the enzymes, either due to the inhibitory effect of low temperature on the activity per se and/or limitations in the enzymatic function.

2. Cold acclimation

Cold acclimation allows the plant to deal with the problems stated above. The process involves both avoidance from and tolerance to freezing (Fig. 2). Freeze avoidance aim to lower the freezing temperature of the tissue, by supercooling along with the accumulation of soluble sugars, preventing ice from forming. Freezing tolerance allow ice to form without lethal outcome, and include changes that helps to protect the tissue. In order to cold acclimate the plant must first perceive the change in temperature, transduce the signal, activating or repressing appropriate genes to ultimately make the changes necessary.

2.1. Perception and cold signaling

An immediate effect of low temperature is a decrease in membrane fluidity (Levitt, 1980) and thus it has been implied that the cold sensor is connected to the plasma membrane. The first evidence came from a study in Synechocystis PCC6803 where chemically induced membrane rigidification caused the induction of a cold responsive gene (Los et al., 1993), a response that later was confirmed in plants by Orvar et al. (2000). Little is known about the actual cold sensors, and how the
conversion of the physical signal is achieved. An early event in response to low temperature is the influx of calcium into the cytosol and the involvement of calcium in cold signaling has been demonstrated in numerous studies (Knight et al., 1991; Monroy et al., 1993; Tahtiharju et al., 1997; Plieth et al., 1999; Orvar et al., 2000). A rise in intracellular calcium has been observed within 10 seconds of cold treatment, inducing cold responsive genes such as RD29A (Nordin Henriksson and Trewavas, 2003). This induction of RD29A is reduced by the addition of calcium signaling antagonists, that also partially reduces the low temperature induced increase in calcium.

The membrane rigidification correlated with the influx of calcium occurs through actin-filament reorganization. By treating Medicago sativa (alfalfa) cells with membrane and actin microfilament stabilisers the influx of calcium and expression of cold-regulated (COR) genes could be prevented (Orvar et al., 2000). This rearrangement of the cytoskeleton may be responsible for the opening of integral stretch-sensitive calcium channels within the plasma membrane. Another important player suggested to be involved in cold signaling is the molecule inositol-1,4,5-triphosphate (IP3). Arabidopsis mutants (FRY1) with increased levels of IP3, show increased induction of low temperature and ABA responsive genes, indicating that it mediates ABA and stress signal transduction in plants. Exogenously applied IP3 results in a release of calcium from vacuolar vesicles and isolated vacuoles (e.g. Schumaker and Sze, 1987) and mediates a transient increases in cytosolic calcium.

![Diagram of plant responses to environmental stress](image-url)
These results all indicate that the initial perception of abiotic stress results is dependent on both calcium and IP3.

It is suggested that the specific calcium signature (kinetics, magnitude, and cellular source) helps the cell distinguishing one stimulus from the other (Sanders et al., 1999). The changes in calcium levels results in alterations in protein phosphorylation within minutes after cold treatment (Monroy et al., 1993; Monroy et al., 1998) suggesting that calcium acts as a secondary messenger in the signal transduction pathway, together with a MAP kinase cascade. Several protein kinases including the Arabidopsis MEKK1 are transcriptionally upregulated at low temperature (Mizoguchi et al., 1996), and the protein interacts with the downstream MAPKK MKK2 (Mizoguchi et al., 1998; Ichimura et al., 2000). Constitutively expressing or overexpressing MKK2 in Arabidopsis results in elevated MAPK kinase activity and improved freezing and salt tolerance, as well as a strong upregulation of several calmodulins and other calcium binding proteins (Teige et al., 2004).

2.2. Gene regulation and expression

In 1994 Yamagochi-Shinozaki and Shinozaki identified a 9-bp DNA element in the promoter of the Arabidopsis RD29A gene. This regulatory element became known as the dehydration responsive element (DRE), containing the core sequence, CCGAC, designated the C-repeat (CRT). This regulatory sequence has since been found to be essential for low temperature responsiveness of several COR genes (Baker et al., 1994; Jiang et al., 1996; Ouellet et al., 1998). Stockinger et al. (1997) isolated the first cDNA clone for a DRE/CRT-binding protein, named CBF1. Since then a number of CBFs has been isolated in Arabidopsis, the genes are referred to as DREB1A/CBF3, DREB1B/CBF1, DREB1C/CBF2. A fourth CBF paralog, CBF4, has been shown to be drought rather than cold responsive (Haake et al., 2002). The CBF-genes are induced within 15 minutes after exposure to low non-freezing temperatures followed by induction of genes that contain the DRE/CRT element. Constitutive expression of the CBF1, CBF3 and CBF4 genes in transgenic Arabidopsis plants results in the induction of COR gene expression and an increase in freezing tolerance without a low temperature stimulus (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Haake et al., 2002). Similarly, overexpression of either AtCBF1 or 3 enhanced chilling and freezing tolerance in Brassica (Jaglo et al., 2001), tobacco (Kasuga et al., 2004) and rice (Ito et al., 2006).

Although the CBF regulon plays an important role in the regulation of the cold acclimation process, it is not the only participant. The eskimo-1 mutant in Arabidopsis is constitutively freezing tolerant without affecting the genes that belong to the CBF regulon (Xin and Browse, 1998), suggesting that there are other regulons important for the regulation of cold induced gene expression. Zhu et al. (2004) has reported the existents of such a pathway mediated by the HOS9 gene product. HOS9 encodes a transcription factor that controls at least 175 genes that
does not appear to be regulated by the CBF regulon. Mutant hos9 plants also show an increased freezing sensitivity both before and after acclimation.

It is clear that the subject is complex and that there are literally many paths to follow, much work is still needed to get a comprehensive understanding of cold sensing and signaling.

2.3. Responses increasing the cold tolerance
Cold tolerant species have developed ways to deal with the dangers of low temperature and adjust in order to better survive freezing temperatures. Some of the changes that take place will be accounted for in the following sections.

2.3.1. Stabilisation of membranes
An initial step in the cold acclimation process is to decrease the sensitivity of the plasma membrane (Steponkus, 1984). This is probably achieved both by changes in lipid composition, which alters the dehydration induced phase behavior of the membrane, and by the accumulation of substances in the surrounding cytosol, interacting with and stabilising the membrane.

Following cold acclimation the proportion of practically every lipid component is altered, when expressed as mol % of the total lipid fraction (Lynch and Steponkus, 1987). The most pronounced changes are an increase in the proportion of phospholipids, due to an increase in the proportion of di-unsaturated types of phosphatidylcholine and phosphatidylethanolamine, and a decrease in the proportion of cerebrosides. These alterations change the cryobehaviour of the plasma membrane and results in an increase in membrane fluidity (Steponkus et al., 1990). The importance of membrane fatty acid unsaturation has been confirmed, *Arabidopsis* mutants with reduced levels of polyunsaturated fatty acids were found to be more sensitive to chilling (Miquel et al., 1993). After cold acclimation the properties of the plasma membrane is altered with freeze-induced contractions resulting in exocytotic extrusions instead of endocytotic vesicles, these extrusions are reversibly incorporated into the membrane again during osmotic expansion (Gordon-Kamm and Steponkus, 1984). Loss of osmotic responsiveness still occurs in cold acclimated leaves but it is no longer associated with the transition to a H_{II} phase. Cold acclimated rye leaves have been subjected to temperatures as low as -35°C without the formation of H_{II}-phase. Protoplasts from cold acclimated rye, oat and *Arabidopsis* has revealed deviations in the fracture plane (fracture-jump lesions) between the plasma membrane and various endomembranes in regions where these are brought into close contact and the occurrence of LOR was found to be connected to this phenomenon (Webb and Steponkus, 1993; Webb et al., 1994; Uemura et al., 1995).

In addition to changes in lipid composition, there are experiments hinting at other mechanisms that potentially alter the cryobehaviour of membranes. Artus et al.
(1996) showed that constitutive expression of COR15a, encoding a chloroplast targeted protein in *Arabidopsis*, enhances the *in vivo* freezing tolerance of chloroplasts in non acclimated plants by almost 2°C, it also had a positive effect on the *in vitro* freezing tolerance of protoplasts. The increase in freezing tolerance was assigned to an increase in membrane stability. In a following study Steponkus et al. (1998) demonstrated that the increased stability was a result of a decreased occurrence of freeze-induced lamellar-to-HII phase transitions. Furthermore, they showed that the protein encoded by COR15a, has the ability to increase the temperature at which the lamellar-to-HII phase transition occur and it also promotes formation of the lamellar phase in a lipid mixture composed of the major lipid species of the chloroplast envelope. From these results the authors suggest that COR15a, push freeze-induced formation of the HII phase to lower temperatures by altering the intrinsic curvature of the inner membrane of the chloroplast envelope. This idea has recently been challenged by evidence localising the COR15a protein exclusively to the chloroplast stroma, raising the question of how the protein can affect the chloroplast envelope if it does not associate with the membrane. The protein was also shown to form oligomers which are capable of interacting with other proteins (Nakayama et al., 2007). The cryoprotective function of the protein was confirmed and there is still the possibility that COR15a work together with other membrane associated proteins that mediates the interaction. The authors, however, propose another function where the protein would activate enzymes or protect enzymes within the stroma from inactivation. This was supported by the fact that purified COR15a was able to protect L-lactate dehydrogenase against freeze-inactivation (Nakayama et al., 2007).

### 2.3.2. Readjustment of carbon metabolism

A well-known response to low temperature is an early and major shift in carbohydrate status of the plant, with both a quantitative and qualitative change in carbohydrate content. The most widespread accumulated free sugar at low temperature is sucrose (Guy et al., 1992). The increase in sucrose occurs in the cytosol rather than the vacuole (Koster and Lynch, 1992) and the increase can be as high as 10-fold. However, sucrose is far from the only solute accumulated, in higher plants, other commonly accumulated compatible solutes are raffinose, glucose, fructose, glycinebetaine and proline. It is not clear whether an increase in sugar content is causally related to the increase in freezing tolerance or if it is merely a low temperature response as there are very few studies of the cryoprotective role of sugars in plants. Nonetheless the ability to accumulate soluble sugars has been shown to be correlated with the capacity to cold harden in *Arabidopsis* wild type and transgenics with altered capacity for sucrose synthesis (Strand et al., 2003) and they have the ability to act as osmolytes, changing the osmotic potential of the cell and reducing the loss of water (Steponkus, 1984). Recent data has revealed that the early increase in sucrose in *Arabidopsis* in response to low temperature precedes the increase in transcription of one of the key
enzymes involved in sucrose synthesis (SPS), showing that the initial build up of sucrose is not dependent on increased transcription (Kaplan et al., 2007).

In addition, a number of in vitro studies indicate that these sugars have the power to stabilise membranes and proteins subjected to freezing or dehydration (Carpenter and Crowe, 1988; Uemura et al., 2003). All cells contain a small volume of water that does not readily freeze, this water is required in order to create the hydrophilic environment necessary to stabilise the lipids within the bilayer. During severe freezing and extensive dehydration the acyl chains become densely packed together to form a highly ordered gel-phase, this change in structure can lead to the fracture-jump lesions mentioned earlier. Uemura and Steponkus (2003) showed that incubation of Arabidopsis seedlings in sucrose solution was connected to the occurrence of various freeze-induced membrane lesions. The concentration of sucrose required to minimize or preclude damage differed between the types of lesions. The occurrence of EIL at temperature ranges between -2 and -4°C was decreased and prevented after incubation in low concentrations of sucrose (10-35mM), while the occurrence of LOR between -5 and -6°C was reduced by moderate concentration of sucrose (30-200mM). At lower temperatures, over the range of -8 to -12°C, higher concentrations of sucrose (100mM-400mM) was required in order to reduce the occurrence of LOR. The increased concentration of sugars and other solutes has been hypothesised to increase the spatial separation between membranes by replacing the water. In vitro experiments has shown that the presence of mono- and disaccharides, in the intermembrane space can decrease the extent of which the membranes are brought close together, and thereby push the incidence of lamellar-to-gel phase to lower temperatures (Wolfe and Bryant, 1999; Koster et al., 2000; Koster, 2001). Apoplastic sugars has also shown to decrease the adhesive energy that develops between hydrated plant surfaces and extracellular ice (Olien, 1992).

Proline is a compatible solute that accumulates in response to a variety of environmental stresses. It is regarded as having multiple roles, acting as a mediator of osmotic adjustments, a stabiliser of subcellular structures, and a scavenger of free radicals but also as a buffer of cellular redox potential. Cold acclimation in wild-type Arabidopsis results in an up to 10-fold increase in proline (Strand et al., 2003). The Arabidopsis mutant eskimo-1, has been shown to be constitutively freezing-tolerant, these plants contain up to 30-fold more proline, 2-fold higher levels of total soluble sugars and a 3-fold higher increase in RAB18, a cold responsive gene encoding a dehydrin, in comparison to wild-type (Xin and Browse, 1998). It has also been shown that transgenic plants with suppressed degradation of proline are improved when it comes to freezing tolerance, supporting the results obtained with the eskimo-mutant (Nanjo et al., 1999). Proline is able to stabilise proteins during freezing in vitro by maintaining a hydration shell of the protein in its native form (Carpenter et al., 1990).
Many species also accumulate raffinose family oligosaccharides in response to low temperature. Similar to the other compatible solutes they help to stabilize the membranes by replacing the lost water, preventing lipid phase transitions (Hincha et al., 2003). Raffinose is synthesised from sucrose by the addition of galactose donated by galactinol, the synthesis of galactinol is considered to be the key regulatory step in the pathway, controlled by the enzyme galactinol synthase (GolS). In Arabidopsis, the increase in raffinose at low temperature has been associated with an increase in transcript (Liu et al., 1998) of one (GolS3) of the seven GolS genes (Taji et al., 2002; Kaplan et al., 2007). The induction is controlled by the CBF transcription factors (Fowler and Thomashow, 2002; Maruyama et al., 2004). Kaplan et al (2007) showed that the increase in raffinose was preceded by its substrates, as well as the transcriptional induction of two of the enzymes within the biosynthetic pathway, GolS3 and raffinose synthase (RS). The increase in transcription was shown to be an early event, peaking after 12h at 4°C after which the expression gradually declined. The increase in galactinol was detected the same time as the peak in expression of the enzymes and the increase in raffinose was seen after another 12h and the increase continued up until last sampling point (96h) when it had increased 45-fold (Kaplan et al., 2007).

However, the role of raffinose in enhancing freezing tolerance is contradictory. Transgenic Arabidopsis accumulating high levels of raffinose, due to an over-expression of one of the drought responsive GolS genes, increased the tolerance against drought, indicating a function in abiotic stress tolerance (Taji et al., 2002). Comparing two accessions of Arabidopsis (col-0 vs C24) with different freezing tolerance, showed that raffinose accumulated to a larger extent in the more tolerant accession (col-0) in both non-acclimated and acclimated state (Klotke et al., 2004). In support of this conclusion, transgenic petunia plants with reduced galactosidase activity, resulting in higher levels of raffinose has been shown to increase their freezing tolerance in comparison to wild-type plants (Pennycooke et al., 2003). On the other hand, Arabidopsis RS knockouts, which are unable to accumulate raffinose, and transgenic Arabidopsis plants with increased expression of GolS, accumulating higher amounts of raffinose in comparison to wild-type showed no difference in freezing tolerance in either their non-acclimated or acclimated state (Zuther et al., 2004). One explanation to this discrepancy is indicated by the fact that the increase in raffinose in the transgenic petunia is brought about through the manipulation of β-galactosidase, an enzymes that has been shown to have a variety of substrates besides the raffinose family oligosaccharides. Another possibility is that freezing tolerance in petunia is related to an increase in stachyose, which is not seen in Arabidopsis, rather than an effect of the raffinose accumulation.

There is still an ongoing debate about the causality between the accumulation of different soluble sugars and osmolytes and cold acclimation. In many cases there is still a lack of knowledge about the functional role and more direct studies beyond mere correlations are needed.
Changes in the Calvin cycle

The Calvin cycle uses the energy captured in the electron transport chain to fix carbon from CO₂, and convert it to organic compounds for further use by the organism. The cycle is autocatalytic, with each compound being both a substrate and a product, and comprised of three phases. In the first phase, carboxylation, CO₂ is combined with a five carbon sugar, RuBP, yielding two molecules of 3PGA. The following step is the reduction of 3PGA to triose-phosphate by the enzymes phosphoglycerate kinase (PGK), NADP-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triose phosphate isomerase (TPI). The third phase, regeneration, refers to the regeneration of RuBP from triose phosphate, a process requiring several steps catalysed by aldolase (Ald), stromal fructose-1,6-bisphosphatase (sFBPase), transketolase (TK), seduheptulose-1,7-bisphosphatase (SBPase), ribulose phosphate epimerase (R5P3ep), ribose phosphate isomerase (R5PI) and finally phosphoribulokinase (PRK). The cycle needs to be tightly regulated in order to prevent the depletion of ATP and NADPH and intermediate pools, this is done through enzymatic control. Rubisco, sFBPase, SBPase and PRK all catalyse irreversible reaction, in addition these enzymes together with GAPDH are highly regulated by light dependent changes in the chloroplast such as reduced thioredoxin, pH and magnesium concentration.

The recovery of photosynthetic capacity in cold acclimated plants is dependent on an increase in the activity of the enzymes within the Calvin cycle. The cold response of eight of the enzymes has been examined in Arabidopsis and after 10 days at 5°C only three of the enzymes showed small increases in activity (Strand et al., 1999). However, in leaves that had been allowed to develop at 5°C all of the enzymes showed an increased, with Rubisco, sFBPase, SBPase, GAPDH and Ald exhibiting at least a doubling in activity. The increase was not due to a specific increase in total activity but rather to a general increase in the total leaf protein that results in an increased activity. A recent study of the chloroplast stromal proteomes of Arabidopsis revealed that there was an increase in abundance, on a plastid protein basis, of Rubisco in response to low temperature, while PGK, GAPDH, sFBPase, R5P3ep and PRK showed a significant reduction relative to the total pool of proteins (Goulas et al., 2006) in agreement with previous result demonstrating changes in enzymatic capacity on a per leaf protein basis (Strand et al., 1999). GAPDH, Ald, sFBPase and SBPase are all situated directly downstream of Rubisco and their products serve as precursors for sucrose and starch biosynthesis, as well as the regeneration of RuBP. An up regulation of the reduction phase supports carbon fixation and end product synthesis, at same time as lower relative activity of the regeneration phase restricts fluxes and decrease sequestering of Pi in metabolites of the regenerative parts. In contrast to Arabidopsis, spring cultivars of wheat and Brassica napus, with restricted ability to cold acclimate, exhibit limited capacity to increase the activity of the enzymes involved in carbon metabolism (Hurry, 1995). Correlated with the enhancement of certain Calvin cycle enzymes in
cold developed leaves is a release in suppression of photosynthetic gene expression in *Arabidopsis* (Strand et al., 1997). These cold developed leaves showed a full recovery of the transcript levels even though they maintain high amounts of soluble carbohydrates. It has, however, been shown that sugar accumulation *per se* is not the signal regulating the expression of photosynthetic genes but the signal may be related to the metabolism of hexose phosphates (Krapp et al., 1993; Jang and Sheen, 1994; Krapp and Stitt, 1995).

- **Changes involving sucrose biosynthesis**
  The strong inhibition of sucrose synthesis plays an important role in the cold induced loss of photosynthetic activity. The inhibition imposed on sucrose synthesis by low temperatures is counteracted during cold acclimation by a fast and strong increase in both the activity and amount of cFBPase, SPS and UGPase (Guy et al., 1992; Hurry et al., 1994; Hurry et al., 1995; Strand et al., 1997; Strand et al., 1999; Ciereszko et al., 2001). *Arabidopsis* leaves that develop at 5°C show a four to five-fold increase in the activity of both cFBPase and SPS, as well as an increase in expression of the genes, supporting the increase in protein (Strand et al., 1997; Strand et al., 1999). The strong increase in activity of these enzymes has also been demonstrated in other cold hardy herbaceous plants such as spinach, oilseed rape, winter wheat and winter rye, supporting the notion that this is a central feature of the cold acclimation process (Guy et al., 1992; Hurry et al., 1994; Hurry et al., 1995). In leaves of *Arabidopsis* the development of freezing tolerance is connected to a shift in the partitioning of newly fixed carbon into soluble carbohydrates rather than starch (Strand et al., 1997; Strand et al., 1999; Hurry et al., 2002; Strand et al., 2003; Lundmark et al., 2006), demonstrating importance of the specific increase of the sucrose biosynthesis pathway. The enhancement of sucrose biosynthesis serves to increase the rates of Pi release and reduce the Pi-limitation of photophosphorylation. Moreover, there are studies linking SPS activity and the rate of sucrose export, suggesting that the up-regulation of sucrose synthesis may be of importance for re-establishing sucrose export to developing sink tissues (Baxter et al., 2003). *Arabidopsis* plants with decreased expression of cFBPase have altered carbon metabolism with a switch from sucrose to starch synthesis as a consequence, resulting in decreased leaf sugar levels during the day that rise at the end of the night due to starch breakdown. In total the decreased expression of cFBPase results in a metabolic profile that is diagnostic of a stimulation of starch synthesis and inhibition of photosynthesis due to decreased recycling of Pi, comparable to what happens at low temperature (Strand et al., 2000). These plants have limited capacity to cold acclimate, almost certainly as a consequence of their inability to enhance sucrose biosynthesis. Wild-type *Arabidopsis* was shown to increase their freezing tolerance down to -7°C after exposure to 5°C for a period of 10 days, whereas the cFBPase antisense plants only reached a freezing tolerance of about -5°C (Strand et al., 2003). Further evidence for the importance of the up-regulation of sucrose biosynthesis is provided by transgenic *Arabidopsis* plants over expressing maize SPS. These plants have higher flux of carbon into soluble
sugars and a significant increase in soluble sugar/starch ratio, they also show significantly less inhibition of photosynthesis when shifted to low temperature (Strand et al., 2003). This improvement of sucrose synthesis resulted in an increased freezing tolerance, reaching -9°C after 10 days at 5°C (Strand et al., 2003).

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**Redistribution of Pi**

Redistribution of inorganic phosphate is important for the recovery of sucrose synthesis. In warm grown leaves of well-fertilized plants cytosolic Pi pool are relatively constant and excess Pi is located within the vacuole (Bieleski and Ferguson, 1983; Foyer and Spencer, 1986; Sharkey and Vanderveer, 1989). The depletion of the Pi-pool at low temperature (Hurry et al., 1994; Hurry et al., 1995; Strand et al., 1999) cause a high demand for accessible Pi in the cytoplasm. Although the total pool of Pi within the cells do not change greatly after cold exposure there are indications of a redistribution from the vacuole towards the cytosol (Strand et al., 1999), increasing the cytosolic Pi pool in the long-term. This indicates that Pi limitation of photosynthesis is not likely to occur after long-term exposure to low temperature. Studies of *Arabidopsis* mutants *pho1-2* and *pho2-1* with decreased and increased leaf phosphate levels respectively provide evidence
for the importance of Pi concentration in the development of cold acclimation, related to photosynthetic carbon metabolism. (Hurry et al., 2000). In cold developed leaves of *pho1-2* the levels of accessible Pi increased several fold and was connected to an increased induction of SPS activity and SPS and eFBPase expression compared to WT, whereas no increase could be detected in *pho2-1*. The repression of photosynthetic genes, after low temperature exposure, was abolished in the *pho1-2* mutant and heightened in the *pho2-1* mutant. In all cold acclimation was improved in *pho1-2* and weakened in the *pho2-1* mutant compared to WT.

- **Changes in the interconversion between starch and sucrose**
  The interconversion of starch to sucrose has received considerable recent attention in connection with the cold-induced sugar accumulation that is believed to enhance the degree of freezing tolerance. The exact molecular mechanism behind the accumulation, especially during an early phase of cold acclimation is unclear. However the conversion of starch into soluble sugars during temperature stress has been a well known fact for a long time (Siminovitch et al., 1952; Parker, 1962; Sakai, 1974). The importance of starch breakdown during early stages of cold acclimation was recently demonstrated using *Arabidopsis* mutants called the *sex1* (starch excess1) mutants (Caspar et al., 1991; Yu et al., 2001). *SEX1* has been shown to encode a starch-related α-glucan/water dikinase (GWD), a protein that facilitates the phosphorylation of α 1→4 glucan chains at the C6 and C3 position (Ritte et al., 2002). Studies in potato and *Arabidopsis* have shown that GWD acts as a global regulator of starch catabolism. The six allelic *sex1* mutants in *Arabidopsis* all show impaired starch degradation in dark-adapted leaves (Caspar et al., 1991; Yu et al., 2001), connected to reduced starch phosphorylation levels (Yu et al., 2001), similar to the results of GWD antisense potato plants (Lorberth et al., 1998). The *sex1* mutants display reduced ability to increase their freezing tolerance within the first 24h of cold exposure (Yano et al., 2005). They fail to accumulate maltooligosaccharides, as well as normal levels of Glc and Fru during the early stages of cold acclimation, but do not show any abnormal phenotypes in the fully cold acclimated state (Yano et al., 2005). A second GWD-like protein (GWD3 or phosphoglucan water dikinase (PWD)) has been discovered in *Arabidopsis*. This enzyme catalyses the same reaction as GWD, and is required for normal starch breakdown (Baunsgaard et al., 2005; Kotting et al., 2005). Unlike GWD, GWD3 does not act on unphosphorylated glucans, suggesting that it acts downstream of GWD (Baunsgaard et al., 2005). Although the mechanism behind the degradation of the starch granule is not fully elucidated, it is clear that phosphorylation of starch is essential for hydrolytic breakdown to take place. It is possible that the α-glucan/water dikinases phosphorylates the amyllopectin making it accessible for the degrading enzymes (Blennow et al., 2002; Ritte et al., 2002). In the chloroplast there are two alternative pathways of further degradation. First, chloroplastic glucan phosphorylase (PHS1) (Zeeman et al., 1998) can catalyse the conversion of terminal glucosyl units to glucose-1-phosphate, which can be converted into triose phosphate and exported out of the chloroplast via the triose phosphate translocator.
(Hausler et al., 1998). The second possibility is that β-amylases hydrolyses the β-1,4-glycosidic linkages of the polyglucan chains at the non-reducing end producing maltose or maltotriose, which are too short to be further metabolised by the β-amylases. There are solid evidence that maltotriose is further metabolised by the disproportionating enzyme 1 (DPE1). In dpe1 mutants of Arabidopsis, maltotriose accumulated in the dark and starch breakdown was retarded (Critchley et al., 2001). DPE1 produces glucose and maltopentose. The glucose residues can be exported out to the chloroplast via the glucose transporter while the maltopentose can be attacked again by β-amylase, producing maltose and maltotriose. In the end the net products of the breakdown of these linear oligosaccharides would be maltose and to some extent glucose. Studies have shown that degradation of linear glucans in Arabidopsis usually takes place via BMYs rather than PHS1. Knockout mutants of PHS1 have normal rates of starch breakdown (Zeeman et al., 2004) while knockouts of one of the chloroplastic BMYs have reduced rates of degradation (Smith et al., 2004; Kaplan and Guy, 2005), similar to what is seen in BMY antisense plants of potato (Scheidig et al., 2002). Arabidopsis has nine BMY genes, including one that has been targeted (BMY8) (Ferreira et al., 2004), and three that are predicted to be targeted, to the chloroplast (BMY6,7,9) (Scheidig et al., 2002). Numerous studies have shown that maltose is exported out of isolated chloroplasts (Servaites and Geiger, 2002; Weise et al., 2004) and the discovery of a maltose transporter located in the chloroplast envelope (Niittyla et al., 2004) brought further attention to this route of carbon export to the cytosol.

Once in the cytosol maltose is proposed to be further metabolised by the disproportionating enzyme 2 (DPE2) to glucose and/or sucrose, and maltodextrins (Chia et al., 2004; Lu and Sharkey, 2004). DPE2 deficient plants show up to a 100-fold increase of maltose in the leaves, together with lower night-time levels of sucrose (Chia et al., 2004; Lu and Sharkey, 2004). The DPE2 reaction is thus likely to be essential in cytosolic maltose metabolism. Under in vitro conditions DPE2 transfers glycosyl residues to glycogen, using maltose as the glycosyl donor (Chia et al., 2004). No glycogen-like glucan has been found in the cytosolic compartment of plant cells, so it is likely that DPE2 uses this highly branched homoglucan as a substitute for an endogenous carbohydrate not yet identified. Similar to DPE2 the cytosolic phosphorylase (PHS2) shows strong affinity towards glycogen. It is possible that these enzymes use the recently isolated water soluble heteroglucans (SHG) as substrates. The most prominent constituents of these SHGs are arabinose, galactose and glucose, their pattern of glycosidic linkages is highly complex with more than 20 different linkages. Both low- and high-molecular weight heteroglucans has been found within the cytosol of mesophyll cells. In vitro assays have revealed that heteroglucans isolated from leaves acts as acceptor for the both the DPE2 and the PHS2 catalysed glycosyl transfer reaction (Fettke et al., 2005; Fettke et al., 2005; Fettke et al., 2006). DPE2 deficient Arabidopsis mutants have been shown to have unchanged cytosolic heteroglucan pools during the light/dark cycle while the wild type pool increased in size in the dark (Fettke et al., 2006).
The structure of the heteroglucons differed, with the mutants having higher glucosyl content, especially in the outer chains accessible to a hydrolytic or phosphorolytic attack. It has been demonstrated that recombinant DPE2 uses maltose in preference to other oligosaccharides in the presents of SHG or glycogen.

β-amylase transcript and/or activity is induced during low temperature stress and connected to an increase in maltose content (Nielsen et al., 1997; Kaplan and Guy, 2004). Shifting Arabidopsis to low temperature results in an increased expression of one of the chloroplastic β-amylase (BMY8), the increase occur as early as 2h after exposure to cold (Seki et al., 2001) and after 12h the expression had increased 14 fold (Sung et al., 2001). The increase in maltose in Arabidopsis was shown to peak after 4h at 4°C, while the transcript levels of BMY8 peaked after 24h, suggesting that the increase in maltose during cold shock conditions is not exclusively caused by increased transcription. Other contributing factors could be increased β-amylase activity and/or decreased DPE2 or MEX1 activity. Maltose has been shown, in in vitro assays, to have the ability to protect proteins and the photosynthetic electron transport chain under freezing stress (Kaplan and Guy, 2004).

**Changes to respiration**

There are numerous physiological studies demonstrating respiratory acclimation in response to a new temperature regime (Atkin et al., 2000; 2002; Bolstad et al., 2003). The potential for thermal acclimation of respiration varies among species and across different populations within species and is suggested to be, to some extent, dependent on the environment to which the plant is adapted (genetically based) (Larigauderie and Körner, 1995; Arnone and Körner, 1997; Tjoelker et al., 1999; Atkin et al., 2000; Loveys et al., 2003). Larigauderie and Körner (1995) found growth at low temperature to result in little or no acclimation in a number of alpine and lowland species, while a number of other species, of the same genera, acclimated. It is unclear whether there are systematic differences in temperature acclimation potential among species of different functional groups, however six of the genera mentioned above showed differences in ability to acclimate. A number of broad-leaved tree species have been shown to have a lower capacity to acclimate when compared to a number of conifers (Tjoelker et al., 1999), suggesting that functional traits might be used to predict the ability to acclimate.

Acclimation to lower temperatures consistently results in increased respiration rates, in comparison to warm grown plants, when measured at a common temperature (Rook, 1969; Körner and Larcher, 1988; Arnone and Körner, 1997; Atkin et al., 2000; Covey-Crump et al., 2002). As with photosynthesis, there is a developmental aspect and two different stages of respiratory acclimation has been identified by Atkin and Tjoelker (2003). The first stage, referred to as type I acclimation, represents fast adjustments that takes place in pre-existing tissues within days following a sustained change in temperature. This type of acclimation
relies on alterations in the cellular machinery already in place and reflects a change in the availability of respiration substrate and/or the degree of adenylate restriction of respiration (Atkin et al., 2000). The second stage, type II acclimation, is changes seen in tissues that develop at the new temperature. As with full acclimation of photosynthesis complete acclimation of respiration appear to require the development of new tissue with altered morphology and biochemistry (Atkin and Tjoelker, 2003). Leaves that develop at a new lower temperature have higher respiration rates in comparison to warm grown plants over a wide range of measuring temperatures (Atkin and Tjoelker, 2003). It has also been shown that cold developed leaves of *Arabidopsis* have higher leaf mass area and higher nitrogen and protein concentration than leaves that developed at warm temperatures (Strand et al., 1997; Strand et al., 1999; Tjoelker et al., 1999), this could lead to an increase in the total amount of proteins invested in the respiratory chain. The cold developed leaves of *Arabidopsis* exhibit increased mitochondria density in epidermal cells and an increase in the cristae to matrix ratio in mesophyll cells, changes associated with an increase in respiratory capacity and respiratory rate (Armstrong et al., 2006). Temperature often has a greater relative affect on the rate of substrate use, by growth, maintenance processes and respiration in itself, than substrate production, altering the steady-state concentration of respiratory substrate. At low temperature this usually results in an increase in soluble sugars in source leaves (Strand et al., 2003; Lundmark et al., 2006), and thus an increased substrate availability for respiration. Although studies have not found sugar levels to be important in respiratory acclimation (Atkin et al., 2000; Talts et al., 2004), changes in sugar concentration may still influence acclimation by affecting gene expression (Sheen, 1994; Koch, 1996).

Acclimation of respiration may be linked to the demand and synthesis of ATP. Like the case with substrates the demand for ATP can be reduced in the cold, resulting in an inadequate supply of ADP, at the same time as the potential for ATP synthesis is limited due to changes in enzymes capacity (Atkin and Tjoelker, 2003). For example Ribas-Carbó *et al.* (2000) found the flux through the alternative oxidase (AOX) pathway to increase in pre-existing tissues after several days of exposure to low temperature, possibly to ensure that the TCA cycle remains active.
under conditions of low ATP demand (Atkin et al., 2005). A third factor that might affect acclimation of respiration is the levels of reactive oxygen species (ROS) (Atkin et al., 2005). Low temperature potentially increase the amount of ROS due to over reduction of the electron transport chain in both chloroplasts and mitochondria (Purvis and Shewfelt, 1993; Purvis, 1997; Møller, 2001; Foyer and Noctor, 2003). ROS in itself is a powerful signaling agent (Wagner, 1995; Karpinski et al., 1999; Foyer, 1997) and could induce pathways causing an increase in enzymes that eventually results in a reduction in ROS, such as AOX (Purvis and Shewfelt, 1993; Wagner and Krab, 1995; Møller, 2001). Overexpressing AOX in tobacco cells reduced the amount of ROS in half, while antisense inhibition increased the production (Maxwell et al., 1999). In support, exposure to low temperature have been reported to result in an increase in AOX protein and/or activity (Vanlerberghe and McIntosh, 1992; Gonzalez-Meler et al., 1999).

Thermal acclimation of respiration is hence dependent upon the plasticity of a number of biochemical, anatomical and morphological traits that contribute to development of metabolic homeostasis.

2.3.4. Recovering growth and development at low temperature
As stated above, the alterations in carbon metabolism, leading to recovery of flux, requires the development of new leaves at the new temperature regime, and only in these leaves are full freezing tolerance able to develop (Hurry et al., 2002; Strand et al., 2003; Ensminger et al., 2006). Thus it is crucial for the plant to maintain its capacity to supply developing sink tissues with carbohydrates and other substrates to promote growth (Strand et al., 2003; Takagi et al., 2003). The suggested link between SPS activity and sucrose export (Signora et al., 1998; Baxter et al., 2003; Ono et al., 2003; Strand et al., 2003) opens up the possibility that the increase in the rate of sucrose synthesis at low temperature not only cause an increase in compatible solutes but also enhance export of sucrose to developing sinks.

Sucrose, and in some species derivates such as raffinose, stachyose and verbascose, is the major form of photosynthetically assimilated carbon transported in plants. The phloem mediates the transport from source to sink and consists of two key components, the sieve elements (SE) and the closely associated companion cells (CC). The two cell types are highly modified and interconnected by numerous plasmodesmata. Phloem loading involves an active accumulation of solutes, against a concentration gradient, into the sieve-element companion cell complex (SE/CCC), resulting in elevated solute concentrations and pressure at the source end of the phloem. The complete physical pathway of sucrose transport from source to sink has not been elucidated for any plant, and phloem loading is not facilitated by one universal mechanism in all plants (Bush, 1993; Van Bel, 1993; Komor, 1996; Rentsch and Frommer, 1996; Turgeon, 1996). There are two principal pathways for delivery of sucrose into the minor veins of the SE/CCC: (1) sucrose from the mesophyll cells enters the cell wall space (part of the apoplast)
and diffuses through the cell wall continuum, and is actively transported across the SE/CCC plasma membrane of the minor veins by means of H⁺/sucrose symporters; and (2) direct symplastic cell-to-cell diffusion via plasmodesmata, maintaining a diffusion gradient by converting the sucrose to larger sugars (raffinose and stachyose) in the companion cells of the minor veins (Haritatos and Turgeon, 1995). The extent to which plants utilize either of the two routes for sucrose delivery depends on the number of plasmodesmata connecting the minor veins of the SE-CCC to its surrounding cells, there are also some questions about whether plants can switch between different pathways.

The SE/CCC of Arabidopsis has a relatively high number of connections to the surrounding cells, but the route proposed for sucrose loading still involves the uptake of sucrose from the apoplast into the phloem by the help of sucrose transporters. The phloem parenchyma cells in the minor veins are of type B (Gunning and Pate, 1969) with cell wall ingrowths restricted to the area closest to the SE and CC. These modified parenchyma cells are suggested to serve as conduits receiving sucrose symplastically from bundle sheath cells and other phloem parenchyma cells and releasing it to the apoplasm along the wall shared with the SE/CCC. The sucrose is then actively retrieved and loaded into the SE-CCC (Haritatos et al., 2000). It is also noteworthy that the plasmodesmata of the minor vein CCs in Arabidopsis, together with Moricandia, are structurally distinct from those of other species examined. They have only a single branch on the CC side (Haritatos et al., 2000) whereas many other species have highly branched connections on the CC side (Turgeon, 1996). In Moricandia these channels have been shown to be obstructed, indicating that diffusion out of the CC is prevented by structural modifications (Beebe and Evert, 1992). Whether this applies to Arabidopsis as well is still unclear.

At present at least 20 different cDNAs encoding disaccharide transporters have been identified from various plant species, named either SUT or SUC (Lemoine, 2000; Williams et al., 2000). All of these transporters belong to the same gene family, and many have been shown to mediate sucrose uptake, functioning as sucrose:proton cotransporters with a 1:1 stoichiometry (Bush, 1992; Lemoine et al., 1996). The proteins fall into three clades, in the case of dicotyledons corresponding to functional or structural differences. Transporters of clade I are generally present in several copies in the genome and have a Michaelis-Menten constant (Km) for sucrose of about 1mM, while the transporters of the other two clades are usually single copies with a much lower Km. Most of the proteins that fall within clade III has an extended central loop with highly conserved sequence (Lalonde et al., 2003). So far nine sucrose transporters have been identified in Arabidopsis, of which seven belongs to clade I, one to clade II and one to clade III. To date, the proteins encoded by five of these have been characterised; they all transport sucrose but they differ in kinetic properties, substrate specificity and expression patterns. AtSUC2 has been shown to be expressed in the companion cells,
predominantly in minor veins (Stadler et al., 1995; Stadler and Sauer, 1996; Weise et al., 2000), while others such as AtSUC5 is expressed in sink tissues like seeds (Baud et al., 2005). Disaccharide transporters are thus likely to have a number of distinct functions in plants.

Antisense studies in potato (Riesmeier et al., 1994) and tobacco (Kuhn et al., 1996; Lemoine et al., 1996; Burkle et al., 1998) have shown that sucrose transport is indeed essential for phloem loading and long-distance transport in these species. The leaves of antisense plants with reduced transport activity contained high levels of carbohydrates. In addition the plants grow at remarkably retarded rates, producing crinkled leaves that exhibit chlorosis and accumulate anthocyanins. In Arabidopsis knockouts of AtSUC2 results in a severely stunted and sterile phenotype (Gottwald et al., 2000). Loading in Arabidopsis has been suggested to be a three step mechanism, involving AtSUC2 together with other sucrose transporters located in the bundle sheath cell and the sieve elements. The cytoplasmic concentration of sucrose increases from the mesophyll towards the sieve element, while the apoplastic concentration decreases. Kuhn (2003) thus thought it reasonable to load sucrose via sucrose transporters with increasing affinity for sucrose, with AtSUT2/SUC3 located in the bundle sheath cells with a Km of about 11.7 mM, and then a transporter with a higher affinity located within the membrane of phloem parenchyma cells, and finally AtSUC2 responsible for loading sucrose into the SECCC. However more recent data has localised AtSUT2/SUC3 to the SE, suggesting that it might be important for retrieval of sucrose along the phloem path (Meyer et al., 2004). Recently the first sucrose transporters targeted to the vacuole were identified in both Arabidopsis (AtSUT4) and barley (HvSUT2) (Endler et al., 2006), which may play an important role in regulating and maintaining cytoplasmic sucrose concentrations and ultimately plant metabolism.

The loss in export capacity has been traditionally been interpreted as evidence that these plants are sink limited and thus experiencing feedback limitations in export. However more recent data has shown that these sucrose transporters might play a crucial role in facilitating phloem loading at low temperature.
Methodological overview

1. Plant material
One of the most widely spread and used model organism in plant science is the small weed *Arabidopsis thaliana* (L) Heynh. It was discovered by, and later named after, Johannes Thal in the sixteenth century. Friderich Laibach initiated the experimental use, concluding that favourable features such as; high seed yield, easy cultivation, fast development, easy crossing with fertile progeny, small chromosome number, possible isolation of both spontaneous and induced mutations, made it ideal for genetic research (Methods in Arabidopsis research, 1992). Although ideal for research, *Arabidopsis thaliana* is a plant species without direct economical value, so why not concentrate the research on a species that is of practical use? The answer is by using a species that has all the benefits, plus the advantage of having the complete genome sequenced, we as scientists can move forward more quickly and spend the resources efficiently. With the knowledge gained we can then move on and apply and test the information on more economically and cultural important species.

During my thesis work I have had the benefit of working both with *Arabidopsis* as well as moving forward applying and testing the knowledge in less classical study organism.

1.1. Arabidopsis thaliana- wild type and transgenics
Paper I and II continues the ongoing work concerning cold acclimation in *Arabidopsis thaliana* (L.) Heynh. In the first paper wild type (ecotype Col-0) and two transgenic lines were used. These transgenic lines have altered capacity to produce sucrose, achieved by targeting the key enzymes of the sucrose biosynthesis pathway. The SPSox plants over-express a maize family B SPS (Lunn and MacRae, 2003), resulting in increased sucrose biosynthesis. In the FBPas plants antisense repression of the cytosolic FBPase resulted in plants with reduced capacity for sucrose biosynthesis and a compensatory increase in starch biosynthesis. The transgenic plants have been described in extent by Strand et al. (2000; 2003).

In the second paper only wild type material of the ecotype Col-2 was used.

1.2. Plants of different functional groups
In Paper III thermal effects on respiration and photosynthesis were tested on plants of diverse functional groups. The functional groups used were forbs, grasses and evergreen trees/shrubs. Nineteen species were selected representing a wide range of growth forms, with divergent leaf mass per area (LMA) as well as respiratory and photosynthetic rates. The grasses used were - *Bromus ramosus*, *B. erectus*, *Poa trivialis* L., *P. cistiniana* J Vicker; Forbs- *Achillea millefolium*, *A. ptarmica*, *Plantago major*, *P. euryphylla*, *Silence dioca*, *S. uniflora*, and *Arabidopsis thaliana* (ecotype Ost-0); Evergreen shrubs/trees- *Acacia melanoxylon* R. Br., *A. aneura* R.
Muell Ex Benth, *Cistus ladanifer* L., *C. laurifolius* L., *Eucalyptus Dumosa*, *E. delegatensis*, *Quercus suber* and *Q. ilex*. Details of the origin and natural distribution of some of these species can be found in Loveys et al. (2002): the rest can be found in Table S1 of Paper III.

1.3. *Crocus vernus*

In Paper IV the attention was turned to *Crocus vernus*. A plant species adapted to take advantage of the short period in early spring when there is a high availability of resources and low competition, as well as to cope with the persistent low temperatures of the season. Since *C. vernus* deposit, and depends on, carbohydrates in the underground corm, it is suitable for studying the temperature effect on the accumulation of biomass in storage tissues. A more comprehensive description of *C. vernus* can be found in Lapointe (2001).

2. Treatment strategies

When working with model organisms it is easy to lose track of the life-history traits associated with them in their natural environment. Most experimental setups do not even set out trying to mimic nature since it is often too complex with too many variables to consider. It is often necessary to simplify things to answer specific questions, and it is important to keep this in mind when applying the knowledge outside the lab. With the further development of statistical tools helping us with experimental design we should be able to move from the classical approach of changing one variable at a time to systems that allows us to deal with the interactions and synergistic effects of several variables.

The majority of studies of low temperature effects have been done by short term shifts to lower temperatures, often spanning a few hours or days. These studies deals mostly with the stress phenomenon, for cold acclimation to take place the plants need to be exposed for longer periods giving them time to make the necessary adjustments. In paper I and III two different treatment strategies were
employed; a short-term shift from warm temperature (23°C or 21°C) to low temperature (5°C - 7°C) representing a stress situation, a long-term shift, to the same temperatures, allowing time for the development of new tissue and full acclimation to occur.

In paper II one set of plants were shifted, from 23°C to 5°C, allowing full cold acclimation to take place, as in the two other papers. In addition, a second set of plants were allowed to germinate and develop in 5°C, completing the entire growth cycle at low temperature.

In Paper IV *Crocus vernus* plants were either been grown at 18/14°C (day/night) or at a lower temperature regime of 12/8°C.

3. Methods

Many of the methods and techniques used within the scope of this thesis are well known and need no further introduction. All material and methods used are described in their respective paper as well.

3.1. 14C incorporation

One of the most useful techniques when studying carbon metabolism is the use of radiolabelled carbon. In Paper I and IV intact plants of *Arabidopsis thaliana* and *Crocus vernus*, respectively, were labeled with $^{14}$CO$_2$ released by the acidification of sodium $^{14}$C bicarbonate. This method enables newly fixed carbon to be traced and the flux of carbon through different carbohydrate pools to be determined, thus giving an idea about the activity of different pathways. By allowing different chase periods information about the rate of incorporation to different pools or tissues is gained.

3.2. Whole-plant and leaf gas exchange

Whole plant gas exchange was measured both in Paper I and II by using a custom built open flow gas-exchange system connected to an infrared gas analyser (IRGA; Li-Cor 6262). This set up was able to house intact *Arabidopsis* plants and made it possible to monitor the uptake and release of CO$_2$ for days. It also allowed the temperature to be regulated such that photosynthesis and respiration could be measured at the respective growth temperatures.

3.3. Metabolomics

In the high throughput era of “omics” metabolomics is a relatively new thing, complementing the already existing omics-related methods in the quest for the generation of system biology models. The term metabolome describes the complete set of metabolites within an organism and metabolomics serves to give a snapshot of the unique metabolic composition that the cellular processes leaves behind at a specific time and under specific environmental conditions. These definitions give
an idea of the challenges of metabolomics, the work of identifying and quantifying every single metabolite within a biological system is rather daunting, especially since many of these compounds have never been reported before and lack quantification standards. A number of analytical approaches have been developed, including metabolic fingerprinting and metabolic profiling. Metabolic fingerprinting provides an overview of a metabolite pattern in a treated state versus control, and relies on multivariate statistic to determine if the overall pattern differs significantly between the two systems or not. Metabolic profiling tries to separate and identify as many as possible of the extracted compounds and requires more in-depth analysis by techniques such as gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry or capillary electrophoresis-mass spectrometry.

Cold acclimation is a very complex process involving a variety of biochemical changes at the level of the metabolome, making it difficult to get a good overview using traditional tools. The use of metabolomics allows us monitor changes in all major pathways in response to low temperature. In paper II we used this method in order to try and separate that changes that occur due to the fact that the plant is being stress from changes that are essential for life at low temperature. The samples were analysed according to Gullberg et al. (2004) by GC/TOFMS.

3.4. Semi-quantitative and quantitative PCR
Quantitative polymerase chain reaction (PCR) is in principle a modification of a traditional PCR. In theory a PCR amplifies DNA exponentially, thus given the number of amplification cycles and the amount of end product it is possible to calculate the initial quantity of genetic material. In order to use this method to quantify the amount of transcript within a sample the messenger RNA (mRNA) is converted to complementary DNA (cDNA) using reverse transcriptase which is then amplified and analysed.

In paper I semi-quantitative RT-PCR was used in order to compare the expression of a number of genes. Gene specific primers, spanning introns to avoid amplification of DNA possible DNA contaminations and 18S RNA primers were used in a multiplex reaction. The 18S was used as a control to be able to compare the transcript even thought the amount of starting material may differ to some extent between samples and to correct for tube to tube variation. The efficiency of the 18S primers were modified by the use of a set of competitive primers resulting in expression levels comparable to those of the gene specific primers used. The reactions were optimized to make sure that the PCR was terminated during the exponential phase. The PCR product was analysed on agarose gels and quantified by measuring the optical density, determining the ratio between the 18S control and the gene of interest.

In Paper II we used quantitative real-time PCR, a more sensitive method than the multiplex RT-PCR, with the increase in product measured after each cycle by the use of fluorescent dyes, in this case CyBR Green.
Result and discussion

1. Effects of altered capacity for sucrose synthesis - a role for metabolite transporters in cold acclimation

In paper I we expand on the previously reported importance of re-programming carbon metabolism in acquiring full freezing tolerance (Hurry et al., 1995; Hurry et al., 1996; Strand et al., 1999; Hurry et al., 2000). Several papers have demonstrated that the changes in metabolism that occur in herbaceous plants rely on the formation of new leaves at low temperature (Strand et al., 1997; Hurry et al., 2000), and thus need to be able to supply developing sink tissues with carbon and other building blocks to support growth. Analysis of carbon fluxes in transgenic Arabidopsis (Signora et al., 1998; Strand et al., 2003), tobacco (Baxter et al., 2003) and rice (Ono et al., 2003) over-expressing SPS links SPS activity with the rate of sucrose export. Given this relationship, it is plausible that the enhancement of sucrose synthesis seen during cold acclimation is of importance for relieving the constraint on export and enhancing translocation to growing sink tissues. This connection made us question the role of different metabolite transporters in the chloroplast envelop in the adjustment of carbon metabolism within source cells, and what role disaccharide transporters play in facilitating phloem loading at low temperature. Transgenic Arabidopsis plants with altered capacity for sucrose synthesis and different abilities to cold acclimate were used to address these questions.

1.1. Linking sucrose synthesis and sucrose export

The overexpression of SPS (SPSox plants) results in plants that consistently fix more carbon during a full photoperiod and partition more of the newly fixed carbon into soluble sugar, across all acclimation phases compared to WT (Paper I, Fig. 2 & 3). At 23°C we estimated, using photosynthesis (Fig. 2) and partitioning data (Fig. 3), that these plants produce around 34µmol g⁻¹ FW more hexose units per day than WT. They also increased export of sucrose out of the source leaves (Fig. 5), and as a result only accumulate an additional 4µmol hexose unit g⁻¹ FW day⁻¹ (Paper I, Fig. 5). The SPSox plants are also able to increase the freezing tolerance beyond that of WT (Strand et al., 2003). In contrast, the FBPas plants, with a reduced capacity for sucrose synthesis, have an impaired ability to fix carbon regardless of temperature (Paper I, Fig. 2). The repression of photosynthesis seen when shifted to 5°C is stronger than that of WT, from which they are unable to recover even in leaves developed at low temperature (Paper I, Fig. 2). Unlike WT and SPSox the FBPas plants are not capable of alter the partitioning carbon towards soluble sugars at low temperature (Paper I, Fig. 3). The limited capacity to produce sucrose also resulted in reduced export of sucrose to developing sinks (Paper I, Fig. 5). In the end the FBPas plants are unable to increase the freezing tolerance to the same extent as WT and the SPSox plant (Strand et al., 2003).
These results make it clear that the enhancement of sucrose biosynthesis has a central role in cold acclimation, and that there is potential for an increased freezing tolerance by an up-regulation of the pathway. The results also confirm the connection between the activity of sucrose synthesis and the ability to export sucrose at low temperature.

1.2. Sucrose efflux out of source leaves

In *Arabidopsis* the export of sucrose from the source leaves involves active phloem loading utilizing high affinity sucrose/proton co-transporters in concert with proton pumping ATPases (Riesmeier et al., 1994; Sauer and Stolz, 1994; Truernit and Sauer, 1995; Kuhn et al., 1999; Gottwald et al., 2000). We demonstrate that the increase in sucrose efflux in the SPSox plants was supported by a significant increase in the expression of one of the sucrose transporters, AtSUC1 (Paper I, Figure 6). AtSUC1 shows high homology with AtSUC2, a sucrose transporter localised to the plasma membrane of companion cells (CC) (Riesmeier et al., 1994; Sauer and Stolz, 1994; Truernit and Sauer, 1995; Stadler and Sauer, 1996), and shown to play a major role in loading sucrose into the sieve element companion cell complex (SE/CCC). The severely stunted and sterile phenotype of AtSUC2 knockouts (Gottwald et al., 2000) suggests that AtSUC1 is unable to completely complement the AtSUC2 function of phloem loading. Like AtSUC2, AtSUC1 is a high affinity sucrose transporter of clade I, targeted to the plasma membrane, however, the cell specific localisation of AtSUC1 is still unclear. It was first reported to be flower specific (Stadler et al., 1999), but was later shown to be expressed in leaves as well (Furuichi et al., 2001; Lloyd and Zakhleniuk, 2004), which we could confirm for source leaves (Paper I, Fig. 6 and 7). It is possible that AtSUC1 acts together with AtSUC2, loading sucrose into the SE/CCC or that it is involved in sucrose retrieval into the sieve element along the phloem path, a function that also has been suggested for AtSUT2/SUC3 (Meyer et al., 2004).

The elevated expression of *AtSUC1* in SPSox plants was further enhanced in response to both short- and long term exposure to low temperature (Paper I, Fig. 7). Low temperature also leads to an increase in transcript in WT and to a lesser extent in FBPas plants (Paper I, Fig. 7). Despite the cold responsiveness of the transporter, export was severely compromised after 10d at 5°C in all genotypes (Paper I, Fig. 5). We argue that the loss of export capacity in stressed leaves could be an effect of damages to the membranes, resulting in membrane leakiness and thereby reduced ability to generate the proton-motive force needed for functional sucrose transport. There might also be problems with incorporation of proteins into the membranes during these stress conditions. Studies of BvSUT1 have demonstrated high turnover rates of both symporter mRNA and protein, indicating high transcriptional control (Vaughn et al., 2002). We find it unlikely that the reduction in export can be explained through sink-limited feedback control, as suggested for BvSUT1, since no reduction in expression was detected.
Leaves, of all genotypes, developed at low temperature maintained high expression of *AtSUC1*. In addition SPSox and WT, but not FBPa, showed increased transcription of *AtSUC2* (Paper I, Fig. 7). The SPSox plants also showed an increased expression of the low affinity sucrose transporter, *AtSUT4*, after cold development (Paper I, Figure 7). *AtSUT4* was recently targeted to the vacuole in mesophyll cells (Endler et al., 2006) and its been indicated to be present in CCs (Schulze et al., 2003). Another current study presented evidence for a vacuolar monosaccharide transporter (TMT) in *Arabidopsis*. *AtTMT1* facilitates glucose uptake into isolated mesophyll vacuoles and the transcription was induced by low temperature. Furthermore, *tmt* mutants are impaired in their ability to accumulate fructose and glucose in response to low temperatures (Wormit et al., 2006). The results indicate that these transporters are likely to play a central role in regulating cytoplasmic to vacuolar concentrations of sugars during low temperature conditions. We further demonstrate that the restored export out of source leaves in newly developed tissues (Paper I, Fig. 5 and Paper II, Fig. 4) is connected to a change in leaf structure (Paper II, Fig. 1). The leaves show an increased vascular density, decreasing the distance between the mesophyll cells and the SE/CCC. These features are discussed in more detail in section 2.2.

We argue that the recovered export is a combined effect of an increase in sucrose transporters, together with the companion-cell specific proton ATP-ase (*AtAHA3*) (Paper II, Fig. 5D) generating the proton motive force needed, and changes in vascularisation, reducing the path from mesophyll to SE/CCC. The changes in membrane properties must also be considered to be of importance.

1.3. Balancing carbon flow between the chloroplast and the cytosol

It is important for the plant to be able to balance the carbon flow between the chloroplast and the cytosol to obtain optimal rates of photosynthesis. If too much carbon is withdrawn from the chloroplast the Calvin cycle risk being depleted of phosphorylated intermediates reducing the regeneration of RuBP. If there is inadequate sucrose synthesis phosphorylated intermediates accumulate and deplete the Pi pool, resulting in an inhibition of ATP synthesis, accumulation of 3PGA and inactivation of Rubisco (Edwards and Walker, 1983). There are two major ways of carbon export out of the chloroplast, the transport of triose-phosphate via the triose phosphate translocator (TPT) (Fliege et al., 1978) and the transport of maltose via the maltose transporter (MEX1) (Niittyla et al., 2004).

When we measured changes in expression of these two genes, as an effect of the transgenic manipulation of sucrose synthesis and in response to low temperature, it became evident that the expression of the transporters was altered, possibly due to the altered carbon status of the plants. The SPSox plants showed a strong upregulation of *AtTPT* at warm growth conditions (Paper I, Fig. 6), supporting the increase in photosynthesis and sucrose synthesis (Paper I, Fig. 2). Previous studies of transgenic potato (Riesmeier et al., 1993; Heineke et al., 1994; Hattenbach et al.,
tobacco (Hausler et al., 1998; Hausler et al., 2000; Hausler et al., 2000) and Arabidopsis mutants (Schneider et al., 2002; Flügge et al., 2003), with altered TPT transport capacity, corroborate these results by demonstrating that TPT have a strong control of the flux of carbon between starch and sucrose and over photosynthesis. The SPSox plants also displayed a significant reduction in three of the four β-amylases involved in plastidic hydrolytic starch degradation (Kaplan and Guy, 2004; Kaplan, 2006) with a concurrent decrease in expression of AtMEX1 (Paper I, Fig.6), in agreement with the change seen in partitioning (Paper I, Fig. 3).

Although the SPSox plants show a reduction in carbon partitioning towards the insoluble fraction, the amount of starch accumulated is similar to WT, a pool which is mobilized during night (Strand et al., 2003). This suggests that the abundance of the AtMEX1 transporter do not restrict nocturnal starch mobilization, at least not in growth conditions with long nights (16h). The decreased sucrose synthesis and the increase in partitioning towards starch in the FBPas plants did not cause any significant change in expression of either AtTPT or AtMEX1 (Paper I, Fig. 6). The unchanged expression of AtMEX1 supports the conclusion that it is not limiting night-time mobilisation of starch. There is still the possibility that AtMEX1 expression is repressed by sugar-sensing signals in the SPSox plants, however there are no studies indicating any such metabolite regulation.

A shift to low temperature results in a distinct reduction in transcript of AtTPT in all genotypes, however the repression was most pronounced in the SPSox plants. The mRNA level recovers with time at low temperature but is incomplete even in leaves that developed at low temperature (Paper I, Fig. 7). The increased availability of Pi and phosphorylated intermediates in both the Calvin cycle and the pathway for sucrose synthesis during cold acclimation (Hurry et al., 1993; Hurry et al., 1994; Strand et al., 1997; Hurry et al., 2000) has the potential to shift the balance between the two processes. One consequence of the strong upregulation of sucrose synthesis at low temperature could be to pull much carbon out of the chloroplast via the TPT, counteracting the positive effect on photosynthesis. The cold responsive changes of the Calvin cycle and sucrose synthesis pathway both require an increased consumption of triose phosphates, and as such can be viewed as competitors for the substrate. There are no evidence to imply that the activity of the TPT is affected by post-translational modification nor have any regulatory metabolites been found, indicating that the TPT may be under transcriptional control, with rapid turnover of both mRNA and protein as shown for sucrose transporters (Vaughn et al., 2002). The level of TPT transcript has been shown to be reduced by growth in the presence of sucrose (Knight and Gray, 1994), suggesting that the transcriptional regulation is affected by the sugar status. Thus, depletion of the Calvin cycle may be prevented by transcriptional regulation of AtTPT at low temperature, as suggested by the expression data.
1.3.1. Rerouting carbon export out of the chloroplast

Recently, substantial progress has been made in unraveling the path for starch degradation in leaves. Antisense TPT tobacco (Hausler et al., 1998; Hausler et al., 2000; Hausler et al., 2000) and Arabidopsis T-DNA insertion mutants (Schneider et al., 2002) have shown that plants are able to increase the daytime starch degradation and bypassing the TPT by exporting carbon either as maltose via MEX1 or as glucose via the glucose transporter. The absence of significant starch build-up during the day in response to short and long term exposure to low temperature, despite considerable daily incorporation of $^{14}$C into the insoluble fraction (Paper I, Fig. 3) suggests that transient starch breakdown represents an alternative route for carbon export out of the chloroplast in the cold. In accordance, the expression and activity of β-amylases involved in hydrolytic starch degradation in the chloroplast increase during short term exposure to low temperature and the main breakdown product, maltose, is one of the first carbohydrates to accumulate (Kaplan et al., 2004; Kaplan and Guy, 2004; Kaplan et al., 2004; Kaplan et al., 2006). We could confirm the enhanced expression of AtBMY8 in response to short term exposure to low temperature as well as demonstrate an up regulation in both gene expression and protein of AtBMY9. The increase in transcript of the genes was sustained in leaves that developed at 5°C (Paper I, Fig. 7), strengthening the notion that mobilisation of transient starch is an integrated part of the changes in carbon metabolism important for cold acclimation.

Fig. 6. Illustration changes in transporter expression as a result of changes in carbon flow in oversps plants growing at 23°C (a) and WT acclimating to 5°C (b).
We conclude that by rerouting daytime export of carbon via the breakdown of starch during the day plants are able to avoid the TPT and thereby retain phosphorylated intermediates and inorganic phosphor in the chloroplast at the same time as they maintain high rates of sucrose synthesis.

2. The interaction between development and stress at low temperature - a further investigation of metabolic alterations

In paper II we focus on differentiating responses that are an effect of low temperature stress from responses specific to “life” at low temperature. Several Arabidopsis accessions are so called winter annuals, germinating in the autumn, over-wintering as rosettes to set flowers in spring, thus completing the entire life cycle at moderate temperatures (Nordborg and Bergelson, 1999). There is a developmental element to cold acclimation that is crucial for achieving full freezing tolerance. Depending on the temperature conditions under which the plant or leaf grows and/or develops major differences can be observed in the transcript profile (Fowler and Thomashow, 2002; Lee et al., 2005; Kaplan et al., 2007), metabolic profile (Browse and Lange, 2004; Cook et al., 2004; Kaplan et al., 2004; Gray and Heath, 2005) as well as in protein levels and composition (Cui et al., 2005; Goulas et al., 2006; Yan et al., 2006).

2.1. Photosynthetic carbon metabolism II

When we compared daily CO₂ uptake of intact plants it became clear that although leaves that developed at low temperature, but originated from plants cultivated at 23°C (CD), showed a strong recovery in photosynthetic capacity, relative to leaves that developed at warm temperatures prior to the cold shift (Paper I, Fig. 2), they assimilate significantly less carbon than warm grown (WG) plants on a fresh mass basis (Paper II, Fig. 2). Furthermore they showed an increase in nocturnal respiration (Paper II, Fig. 2) and as a result only acquired approx. 50% of the amount of carbon that WG plants did. In contrast, the cold-grown (CG) plants, which were cultivated at 5°C, showed similar rates of photosynthesis and respiration at 5°C as the WG, showing complete homeostasis of both processes. These changes in whole plant carbon exchange were correlated with alterations in the diurnal pools of different carbohydrates (Paper II, Fig. 3). In agreement with previous studies (Strand et al., 2003) the CD leaves accumulated large pools of carbohydrates. This well established accumulation was abolished in plants cultivated at low temperature, in contrast, they exhibited normal levels of hexoses and transient starch (Paper II, Fig. 3A-B,G).

The metabolome of Arabidopsis is known to be substantially re-organised during cold acclimation (Browse and Lange 2004) and metabolomic studies have shown several hundred of metabolites to be significantly changed in response to ‘cold-shift’ experiments of various durations (Cook et al. 2004; Kaplan et al. 2004; Gray and Heath 2005). Our study confirms that substantial rearrangements of the
metabolome occurs in response to low temperature, PCA analysis reveals that there is a clear separation between the metabolome of plants subjected to low temperature stress and the metabolome that is the result of constant growth at low temperature (Paper II, Fig. 6).

We further analysed the data by projection to latent structures-discriminant analysis (PLS-DA) to maximize the information related to the differences between the classes of samples. In order to identify metabolites associated with cold stress we compared CD with CG/WG samples. The comparison revealed that the relative concentration of 58 putative metabolites differed (Paper II, Table 1), with an increase in a wide range of soluble carbohydrates as well as in organic acids in CD leaves. Roughly the same amount of metabolites was found to change when comparing WG to CD/CW (Paper II, Table 2), revealing a well known increase in proline, maltose and the two main hexose phosphates in response to cold. At last we compared CG to CD/WG in order to identify metabolites were part of the cold adapted metabolome (Paper II, Table 3). In general the CG metabolome shows a stronger resemblance to the WG than to the cold developed metabolome. In all, the data show that some of the metabolite changes that have been associated with cold acclimation appear to be a response to a cold shift or cold stress rather than an acclimation response.

Raffinose, together with associated myo-inositol, were found to be considerably increased in response to low temperature stress (CD) (Paper II, Fig. 3D, Table 1). Consistent with earlier reports (Taji et al., 2002; Cook et al., 2004; Klotke et al., 2004; Kaplan et al., 2007), the build up in raffinose was supported by increased expression of one of the isozyme catalysing the key regulatory step of the raffinose synthesis pathway, galactinol synthase (AtGolS3) (Paper II; Fig. 5B). However, unlike Kaplan et al. (2007) we do not detect any increase in raffinose synthase (RS) transcript in the CD plants, suggesting that it is transient (Paper II, Fig. 5B). AtGolS3 was found to be upregulated in CG leaves as well (Paper II, Fig. 5B), although to a lesser extent than in CD plants, and it did not translate into increased levels of either raffinose or myo-inositol (Paper II, Fig. 3D,F). Sucrose was found to be positively correlated with both CD and CG, even if the accumulation was smaller in CG plants (Paper II, Fig. 3C). The increase was supported by a significant increase in expression of AtSPS5A and AtSPS5B in both CD and CG. The induction of AtSPS5B was especially accentuated (Paper II, Fig. 5B), a previous study reported SPS5B to be more strongly induced during the first 48h of cold exposure after which the amount of transcript for the two genes was found to be similar for duration of the cold treatment which lasted a total of 96h (Kaplan et al., 2007). The expression of AtSPS5A was approx. 2 times higher in CD plants compared to CG, an increase confirmed to result in elevated amount of protein. We were also able to demonstrate a small increase in SPS4 protein, although the increase in expression was not significant. Unfortunately we lack a specific antibody for SPS5B. The data imply that the SPS family A genes in Arabidopsis
are cold responsive. Transcriptional analysis have shown that family A genes from kiwi (Fung et al., 2003), *Vicia faba* (Weber et al., 1996) and citrus (Komatsu et al., 1999) are developmentally regulated. In kiwi, one of the family A genes, were more highly expressed in older leaves than in younger, as well as in tissues associated with starch mobilisation (Fung et al., 2003). The same study also demonstrated that the B family gene responded to low temperature. In leaves of *Arabidopsis* the single gene of family B, *AtSPS1*, has been shown to be expressed in low levels in comparison to the other genes, and we were unable to detect any transcript of the gene in source leaves regardless of growth temperature.

Despite the contrasting accumulation of soluble carbohydrates there was no difference in freezing tolerance, both plant types showed an increase in LT$_{50}$ of about 6°C compared to WG plants (Paper II, Suppl. Fig. 6). In conclusion it seems like although soluble sugars such as glucose and fructose have been shown to act as compatible solutes they are part of a stress response pathway rather than a low temperature specific accumulation. This is may also apply to the accumulation of raffinose. As mentioned earlier its involvement in the enhancement of freezing tolerance has been questioned. Zuther et al. (2004) compared transgenic plants with either increased or abolished accumulation of raffinose and found no difference in the freezing tolerance in either the non-acclimated or acclimated state. They consider the discrepancy between their result and those of Petunia, where the increase in raffinose was correlated with the freezing tolerance (Pennycooke et al., 2003), to be due to the fact that the raffinose accumulation in their experiment was considerably lower. The amount of raffinose accumulated in the CD plants is substantially higher, while the amount in the CG plants is in the range, of those reported for cold treated leaves of transgenic *Arabidopsis* overexpressing *GolS* by Zuther (2004). Thus we can conclude that although raffinose might be of importance for managing the effects of low temperature stress, such as membrane protection, it is not essential for increasing the freezing tolerance in *Arabidopsis*.

Sucrose stands out as one of the sugars accumulated in both CD and CG plants (Paper II, Fig. 3C), suggesting that its accumulation is due to the decrease in growth temperature and not only a stress response. By using the same transgenic plants as in Paper I, Strand et al. (2003) was able to demonstrate a strong correlation between the sucrose concentration and the degree of freezing tolerance. As discussed earlier an upregulation of sucrose synthesis is important for recovering and maintaining photosynthesis and for sustaining growth at low temperature. In addition its role as a compatible solute has been strengthened by recent studies (Cacela and Hincha, 2006; van den Bogaart et al., 2007), demonstrating that sucrose has the ability to bind with the polar groups of lipids in a way that is qualitatively very similar to that between water and lipid. The interaction was found to be weaker, but even so sucrose was shown to depress the phase transition temperature of phospholipids beyond that at full hydration, it is likely an effect of the ability of sucrose to bridge more lipids than water (Cacela
and Hincha, 2006). This feature is probably responsible for causing the decrease in lateral movement of the lipids, connected to increased membrane stability as well. Sucrose also decreased the movement of lipids to a larger extent than other sugars such as fructose, glucose, trehalose, maltose, maltotriose and maltotetrose (van den Bogaart et al., 2007).

2.2. Sucrose export out of the source leaves II
Since sucrose export, is essential to sustain growth of new tissues, at the same time as being strongly affected by low temperature we compared the efflux rates between the different growth regimes. There was a reduction in export capacity in leaves of both CD and CG plants, relative to WG (Paper II, Fig. 4). However, both were able to maintain high rates of sucrose efflux throughout the photoperiod, in contrast to cold shocked leaves (Paper I, Fig. 5). In comparison to WG leaves we found a clear increase in vascular density, resulting in a decreased areole size, across both CD and CG leaves (Paper II, Fig. 1E-F). Although the organ- and species-specific vascular tissue organisations are highly reproducible, the vascular system in dicots displays a high degree plasticity and flexibility (Sachs, 1981 and references therein; , 1989). Arabidopsis leaves have a hierarchical network of veins starting with a central primary vein, followed by successive secondary veins and finally higher-order veins “forming” the areoles and terminating the vein system (Selstam et al., 1992; Kang and Dengler, 2004). The spatial regularity of vascular strands is one of the basic features of the vein pattern of the leaf and of great importance for establishing efficient transport of water and nutrients throughout the plant. In Arabidopsis leaves grown under low light (250µmol m⁻² s⁻¹) the maximum distance from mesophyll cell to vein is around 330µm, and it is estimated that photoassimilates have to move through six or seven cells to reach a vein, a distance common to other species (Haritatos et al., 2000). Vein density has a high intraspecific variability, and studies have revealed connections between venation and a number of environmental parameters. Shade leaves have lower density than sun leaves (Esau, 1965), reduction in soil water availability, along with wind speed (Grace and Russell, 1977) and nutrient deficiency (Philpott, 1956), leads to an increased density. We are able to show that this also applies to long term exposure to low temperature. The increase, at low temperature, was in high order veins, responsible for phloem loading, reducing the distance from mesophyll to vein and thus increasing the likelihood of transport.

The changes associated with the recovery of efflux rates in CD leaves shown in Paper I, along with an increase in vascularisation, could be confirmed for the CG plants as well and therefore appears to be a necessary adaptation to enable sucrose transport in the cold.
2.3. Mobilisation of starch

In contrast to CD leaves, that show a typical accumulation of starch in response to low temperature, the CG plants fully metabolize their starch pool at night similarly to WG plants (Paper II, Fig. 3G). The CD plants also metabolise the transient starch that is built up throughout the day, however they maintain a large static pool that is not metabolised at night. In Paper I we showed that to facilitate the increase in sucrose biosynthesis, necessary for releasing the inhibition of photosynthesis, without jeopardising the regeneration of RuBP, *AtTPT* was strongly repressed and carbon rerouted out of the chloroplast via transient starch degradation. Cold acclimation enables leaves developed at low temperature (CD leaves) to regain a balance between the processes of the chloroplast and the cytosol. The increase in *AtTPT* transcript seen in CD leaves compared to cold stressed leaves (Paper I, Fig. 7) suggests that once the readjustments of carbon metabolism have been completed the need to control export out of the chloroplast is not as strong. This being said the CD plants still display a large increase in expression of genes involved in starch breakdown. The expression of *AtBMY8* and *AtBMY9* as well as genes acting upstream such as SEX1, which is a global regulator of starch catabolism, and GWD3 were strongly induced (Paper II, Fig. 5A). Previous results have shown that the increase in *BMY8* is an early event (hours), connected to a strong and fast increase in maltose (Kaplan and Guy, 2004). We show that the increase in maltose is sustained even after long term exposure to low temperature (Paper II, Table 2). Plants grown from seeds at low temperature show a notably similar response in gene expression as described for the CD plants, with mRNA levels of *AtTPT* slightly lower than WG and a concurrent increase of genes involved in chloroplastic starch breakdown, correlated increased levels of maltose. The increase in maltose could act as a compatible solute, and/or it could simply be an effect of increased daytime starch breakdown. The fact that the accumulation precede the induction of *BMY* transcription suggests that increased gene expression is not the sole cause for the build up (Kaplan et al., 2007). The rapid increase could be an effect of increased β-amylase activity as is suggested by results from potato and/or a result from a decrease in *DPE2* or *MEX1*. There was a slight repression in *DPE2* in CD leaves which was stronger in CG leaves (Paper II, Fig. 5B).

The similar response of the cold developed and cold grown plants indicates that enhancement of chloroplastic starch breakdown is not only a way to deal with an immediate stress but a requirement for enhancing sucrose synthesis while at the same time maintaining functional photosynthesis at low temperature.

3. Thermal acclimation potential and biochemical responses among different functional groups

Global warming is not a new phenomenon, temperature changes has occurred in the past forcing plants to adapt to temperatures higher than those at present day. The novelty this time around is the rate at which the temperature is expected to
increases (Flenley, 1998). A range of credible scenarios predicts a temperature increase of between 1.4 and 5.8°C between 1990 and 2100 (IPCC, 2001). Both photosynthesis and respiration are highly temperature dependent processes and will have an immense effect on the global carbon balance in light of the present global warming (Berry and Björkman, 1980; Larigauderie and Körner, 1995; Atkin et al., 2000; Yeh et al., 2000). Numerous studies have demonstrated that species differ in their capacity to acclimate photosynthesis and respiration to new temperature regimes (Larigauderie and Körner, 1995; Arnone and Körner, 1997; Atkin et al., 2000; Loveys et al., 2003), complicating the modeling of the changes on a wider scale. Most current models do not consider the acclimation potential of these metabolic processes. Fast growing herbaceous plants are known to have a high ability to acclimate and this ability relies on a number of biochemical changes to take place (Hurry et al., 1995; Atkin and Tjoelker, 2003). Until now no studies have investigated whether there is a systematic unity in the thermal acclimation potential among species of contrasting functional groups or if there are differences in the changes required for acclimation. We used three different functional groups; grasses, forbs and evergreen trees/shrubs, representing a wide range of leaf mass per area (LMA), respiration and photosynthetic rates, to address these questions.

3.1. Effects of temperature - systematic differences
The species chosen proved suitable since up to 30% of the variation in respiration and photosynthesis between the species was due to the fact they belong to different functional groups (Paper III, Table 1). However, despite the wide range of leaf structure and carbon economy (Paper III, Table 1) the overall results suggest that there are no systematic differences in the response to temperature or in the potential to acclimate. This indicates that however disparate the absolute rates of photosynthesis or respiration were, the acclimation response is strikingly similar across the different groups of species (Paper III, Table 1 and Fig. 1). Although interspecific variation in the ability to acclimate in response to short term changes in temperature have been reported (Larigauderie and Körner, 1995; Strand et al., 1999; Tjoelker et al., 1999), a more recent study of the thermal acclimation potential of respiration in roots showed no systematic difference between species and/or different N availabilities (Atkinson et al., 2007). In concurrence with these results we suggest that this holds true for respiration and photosynthesis in the species of the different functional groups tested within this study as well.

3.1.1. Acclimation of photosynthesis and respiration
Since no differences were found the temperature responses of the different species were pooled in subsequent analysis in an attempt to define some general principles of acclimation photosynthesis and respiration.

A 10 days shift to 7°C, invoking phase I acclimation (Atkin and Tjoelker, 2003), resulted in a repression of photosynthetic rates in all functional groups (Paper III,
Table 2, Fig. 2 and 4). Shifts to 14°C had less effect and shifts to a higher temperature (28°C) provoked no significant change (Paper III, Fig. 2). As discussed cold acclimation in *Arabidopsis* is dependent on the development of new tissue, so called phase II acclimation. Since this is such a prominent feature we assessed if it applied to other species as well. Although leaf development at 7°C did not lead to complete homeostasis a substantial recovery of photosynthesis could be detected (Paper III, Fig. 2 and 4).

In contrast to photosynthesis the rates of respiration increased with decreasing temperatures, leaves shifted to 7°C had rates 60% higher than control plants when measured at a common temperature of 21°C. Despite this increased capacity the *in situ* rates were repressed (Paper III; Fig. 4) under these cold shock conditions. Once again a shift to higher temperatures had no significant effect (Paper III, Fig. 2). The respiratory rates recovered considerably, in all functional groups, although as for photosynthesis homeostasis was not complete (Paper III, Table 1, Fig. 1, Fig. 2 and 4).

Comparing the responses to short and long term changes in temperature illustrates a connection between the development of new tissue and the degree of acclimation, thus indicating that this feature is not only restricted to herbaceous plants but can be expected to be of importance in very contrasting species.

The development of new leaves has also been linked to restored ratios between respiration and photosynthesis (R:A ratio) (Ziska and Teramura, 1992; Dewar et al., 1999; Loveys et al., 2002; Loveys et al., 2003; Armstrong et al., 2006). We found the recovery of photosynthesis to be less complete than the recovery of respiration and as a consequence the ratio was approximately two times higher than in the controls (Paper III, Fig. 5 and 6). The discrepancy between the studies may be explained by differences in growing temperatures and shifting strategy as previous studies used a higher temperature range not including chilling temperatures. Our results is supported by studies in *Arabidopsis* where the recovery was also shown to be incomplete (Savitch et al., 2001; Strand et al., 2003). Similarly, studies have shown that the balance is not restored in plants exposed to very high average day temperatures (Loveys et al., 2003; Armstrong et al., 2006; Atkinson et al., 2007). Taken together it suggests that acclimation of the R:A ratio is possible over a broad range of temperatures but once pushed beyond a certain optimum temperature range for a given species full recovery can no longer be achieved.

3.2. Biochemical adjustments - a universal response
A number of parameters were investigated to determine if the biochemical underpinnings of the acclimation process are similar among the functional groups. The amount of soluble carbohydrates accumulated in response to low temperature was found to diverge between functional groups with forbs accumulating more
sugars than the other groups, however there were no strong correlation with the degree of photosynthetic or respiratory acclimation (Paper III; Tables 2 & 3). This concur with previous results where sugar concentrations were found not to be vital for respiratory acclimation (Atkin et al., 2000; Talts et al., 2004). There is still the possibility that the change in sugar concentration influence acclimation by means of gene expression. The initial repression in photosynthesis was accompanied by a reduction in the amount of D1 protein of PSII (Paper III, Fig. 7a) which is expected as growth and the use of reducing power is more limited than light capturing and the production of energy at low temperature. This creates an imbalance causing photoinhibition, demonstrated by the drop in Fv/Fm, seen in most of the species, which is later recovered in leaves developed at 7°C (Paper III, Fig. 3), together with restored levels of the D1 protein (Paper III, Fig. 7a). The concentration of Rubisco increased in short term shifted leaves, with a two fold increase at 14°C and an almost three fold increase at 7°C (Paper III, Fig. 7b). The levels remained higher in cold developed leaves compared to control, consistent with studies in both *Arabidopsis* and rye (Hurry et al., 1994; Goulas et al., 2006). Goulas et al. (2006) showed an increase in free ATPase subunits in the stroma of *Arabidopsis* leaves shifted to 5°C, indicative of loss of functional ATPase complexes in agreement with the loss of photosynthetic capability. We found no significant response of the β-subunit of ATPase after a 10 days shift, whereas cold development leads to a significant increase (Paper III, Fig. 7c). Since the dark reaction of photosynthesis is particularly sensitive to low temperature an increase in the enzymes involved is necessary for recovering flux through the pathway.

A part from substrate availability, respiration may also be determined by the availability of adenosine triphosphate (ATP), and ATP synthesis is compromised (Atkin and Tjoelker, 2003). The observed increase in respiratory rates in cold acclimated leaves could therefore be an affect of reduced adenosine restriction, either as a result of increased turnover of ATP, increased ADP concentrations and/or an uncoupling of electron transport from proton transport across the inner mitochondrial membrane. This uncoupling could be assisted via an increase in alternative oxidase (AOX) activity. This would prevent over-reduction of the electron transport chain by increasing the oxidation of excess redox equivalents (Purvis and Shewfelt, 1993; Millenaar et al., 1998) and help reduce the increased production of reactive oxygen species (ROS) that inevitably occur at low temperature. It has been shown that inhibition of AOX results in an increase of ROS (Popov et al., 1997) while overexpression results in a marked decrease. AOX may also prevent inhibition of the TCA cycle under conditions where ADP concentrations are low and proton pumping is restricted, sustaining the supply of carbon skeletons for biosynthesis. The importance of this pathway was supported by a detected increase in AOX protein in cold developed leaves of the herbaceous species within this study (Paper III, Fig. 7e) and by previous reports of increased levels and/or activity of AOX in response to long
term low temperature exposure (Vanlerbergh et al., 1995; Gonzalez-Meler et al., 1999; Ribas-Carbo et al., 2000). We found *Arabidopsis* to deviate from this trend, showing an increase in cytochrome oxidase (COX) rather than AOX (Paper III, Table S2). The increase in COX is consistent with an increase in activity of the cytochrome pathway in isolated mitochondria of low temperature treated *Arabidopsis* plants (Armstrong et al., 2006). The differences in response may be linked to the growth “capacity” and the cold tolerance of the species. *Arabidopsis* is fast growing and cold tolerant, and it is possible that the recovery of respiration is associated with recovered ATP synthesis and/or use in these types of species, whereas slow-growing and/or less cold tolerant species, with less demand for ATP, would rely on an increase in AOX activity.

The impacts of recent climate changes are already evident with geographical distributions of species shifting towards the poles and higher altitudes and phenological events occurring earlier. We can anticipate more of these types of changes to occur in the future. Photosynthesis and respiration has a profound influence on the atmospheric CO$_2$ concentration. Terrestrial plants release approx. 60Gt of carbon into the atmosphere each year, exceeding the release by burning of fossil fuels about ten times (Amthor, 1997; Raich et al., 1997; Field, 2001). The release by plant respiration is naturally balanced by photosynthetic carbon uptake, and consequently the balance between them, on an ecosystem level, determines whether an ecosystem is a sink or a source. Understanding the thermal effect and acclimation potential of plant respiration and photosynthesis will be increasingly important for determining plant performance and thus future atmospheric CO$_2$ concentrations. Global changes in the climate are postulated to result in a loss of biodiversity. The importance of biodiversity can be discussed at length, however in scope of this thesis, studies have demonstrated a reduction in ecosystem biomass as a result of decline in species richness (e.g. Hector et al., 1999; van Ruijven and Berendse, 2005) which may ultimately result in reduced overall plant productivity (Tilman et al., 1996; Symstad et al., 1998) and reduced CO$_2$ fluxes.

In conclusion, we demonstrate that the temperature response of respiration and photosynthesis is dynamic and acclimation occurs in response to new temperature regimes. Thus, when predicting the effect of global changes in temperature on carbon metabolism it is important to take thermal acclimation into account. We show that the underlying biochemical adjustments and ultimately the acclimation potential are remarkably similar between different functional groups making predictions somewhat easier. However, even if the ratio between respiration and photosynthesis is not significantly affected by functional group, we showed that the ratio can not be assumed to be constant across all temperatures as is often the case in present models. Our results show that the ratio is temperature dependent and that homeostasis is not always achieved.

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4. Temperature effect on biomass accumulation in storage tissue of *Crocus vernus*

During periods with low temperatures many plants are restricted in their ability to grow, and in general make their best just to endure. However, there are, as always, exceptions. Deciduous forest spring ephemerals and many spring bulbous species such as *Crocus* and tulips are reliant on and adapted to take advantage of the short period in spring that, although abundant in nutrient and light is generally considered to be unfavorable due to the persistent low temperatures. During this period these plants are committed to complete their above ground (epigeous) growth and ensure that they build up sufficient storage of carbon in the perennial organs to sustain continued growth below ground and to secure survival to the next growing season as well as to secure the reproductive success. Low growth temperature has, in fact, a positive effect on leaf longevity and the final biomass of the underground storage tissue of spring ephemerals and spring bulbous species, including *Crocus vernus* (DeHertogh and LeNard, 1993, Lapointe and Lerat, 2006, Nault and Gagnon 1993, Badri et al., 2007). The enhanced growth has thus far only been reported in a limited number of temperate higher plants (Badri et al., 2007). It is still unclear how temperature influence the growth of the corm of *Crocus vernus* and in Paper IV we try to determine if different aspects of carbon metabolism such as carbon fixation, allocation and partitioning was altered, and if these parameters could help explain why cooler temperatures promotes corm growth.

4.1 Carbon fixation

We found plants grown at 12/8°C (day/night) to develop corms with up to 88% higher final biomass than plants grown at 18/14°C (Paper IV, Fig. 1), confirming previous results (Badri et al., 2007). The optimal temperature of photosynthesis is known to be low in these species (Mamushina and Zubkova, 1996) and in accordance carbon fixation was not repressed at the cooler temperature (Paper IV, Fig. 2). The ability to maintain photosynthetic rates at lower temperatures has been related to an increased protein content (Grime and Monforth, 1982) and increased capacity for sucrose synthesis (Mamushina and Zubkova, 1996). The data suggests that *C. vernus* has the ability to sustain functional photosynthesis over a range of temperatures and we found no divergence in carbon fixation that could explain the difference in growth of the corm.

4.2 Carbon translocation

We traced the newly fixed carbon from the leaves to the corm to determine if temperature had an effect on the translocation of carbon. The initial rate of export was found to be slightly higher at warmer temperature and although the difference was not significant, the amount of carbon that on average reached the corm, after 48 h chase, was clearly higher in these plants (Paper IV, Fig. 3). Since the amount of carbon fixed by the leaves did not differ the effect that growth temperature had on export cannot explain the dimished growth of corms from warm grown plants. If
anything it would imply that the lower temperature restricts the export of carbon, a

tendency discussed previously within this thesis. In general translocation rates have

been shown to be relatively insensitive to temperature with the exception of short
term cold shocks (Minchin et al., 1983) and are generally restored as the plant cold
acclimates (Lundmark et al., 2006).

4.3 Carbon partitioning within the corm

The carbon transported to the growing corm has to be converted into starch if it is
to be stored for later utilization. The soluble sugar pool, comprised of mainly
sucrose and glucose, was the first to be heavily labeled (Paper IV, Fig. 4 & 5), as
expected since C. vernus is likely to transport carbon as sucrose (Lapointe,
personal communication). Partitioning of the total pool of labeled carbon within the
corm differed between the two temperature regimes (Paper IV, Fig. 6f). At 18/14°C
the partitioning towards soluble sugars remained stable around 40% throughout the
entire growth period, while plants grown at lower temperatures increased the
allocation into this pool with each phenological stage, starting with 31% and
ending with 56%. The partitioning into starch was greater at lower temperature in
the beginning of the growth period, with about 65% of the carbon being
incorporated and then decrease steadily to less than 40% in the end of the growth
period. At higher temperature the starch pool contained about 50% of the total pool
of labeled carbon at all phenological stages. Clearly growth at higher temperature
do not reduce the ability or tendency to convert the carbon translocated to the corm
into starch, it is rather promoted in comparison to plants grown at 12/8°C, with the
exception of the initial growth phase. However, the rate of starch accumulation was
comparable between the two temperature regimes (Paper IV, Fig.7e).

In all, these data on carbon fixation, translocation and partitioning together with the
rate of starch accumulation suggests that corm growth at the higher temperature is
not limited by carbohydrate availability. This notion is supported by studies in
onion, that also exhibits reduced growth at higher temperature (Daymond et al.,
1997; Wheeler et al., 2004).

4.4 The dependence of corm size on cell size

In spring ephemerals the replenishment of the carbon storage occurs
simultaneously with cell growth (Badri et al., 2007), and in order for the corm to
store large amounts of starch it has to be able to increase sufficiently in size. Plant
growth can be divided into growth by proliferation and by cell expansion. During
the first phase cell numbers increase through mitotic cycling and the increase in
mass is a consequence of increased cytoplasmic volume which is dependent upon
active protein synthesis and metabolism. The second phase is driven by water
uptake and controlled by modifications of the cell wall, and accounts for most of
the increase in mass. Since organ size reflects both cell number and cell size the
effect of growth temperature on the rate of cell division (Francis and Barlow, 1988)
and/or cell expansion (Pollock and Eagles, 1988) could help explain the discrepancy in corm size between the two temperature regimes. Given the sessile and light-dependent lifestyle, cell growth in plants is often influenced by environmental cues. Pathways regulating growth by cell division generally affects the extent of the period of cell proliferation, rather than the rate of growth. Thus, growth commonly occurs at the maximal rate permitted by the environmental conditions and differences in size are determined by allowing growth to occur for shorter or longer periods of time. However, the control of organ size is further complicated by the fact that morphogenesis is flexible, adapting to changes in the environment, requiring a complex interplay between endogenously and exogenously generated signals. Determining how the coordination of developmental and environmental signals regulate growth has proven difficult (Walch-Liu et al., 2000; Thingnaes et al., 2003; Kozuka et al., 2005; Anastasiou and Lenhard, 2007). Many manipulations of the cell cycle resulting in fewer cells are counteracted by an increase in cell expansion (Discussed in Tsukaya, 2005; Ingram and Waites, 2006). Cell division has been shown to be heavily dependent on temperature (Francis and Barlow, 1988), while cell elongation is considered less sensitive. However, the temperature response is not uniform across all plant species, some have a reduced cell size when grown at lower temperature while others show an increase (Körner and Larcher, 1988; Ben-Haj-Salah and Tardieu, 1995; Tonkinson et al., 1997).

Badri et al (2007) demonstrated that growth at low temperature resulted in an increased final cell size in corms of *C. vernus* compared to plants grown at a warmer temperature regime. At the beginning of the growth period the rate of cell expansion was higher at the higher temperature regime, however growth was arrested already 36 days into the epigean growth period while the cell size continued to increase at lower temperature until the end of leaf senescence. Cell expansion is dependent on the balance between the hydrostatic pressure generated within the vacuole and the physical restraint by the cell wall (Volkenburgh, 1999; Cosgrove, 2000). In agreement with these results we found that the partitioning of carbon into cell wall material within the corm of the warmer grown plants increased earlier (Paper IV, Fig. 4c & h) suggesting that the cell walls become thicker over time causing a restriction in cell expansion.

Although sucrose is the main product of photosynthesis and the main form of transported carbon in most plants, many of the sugar signals that effects growth and metabolism is mediated through its hydrolytic hexose products, or their downstream intermediates. We found the concentration of glucose to decrease drastically early on, in the corm of plants grown at 18/14°C, while the concentration decrease at a much slower rate in plants grown at 12/8°C (Paper IV, Fig. 7). In relation to cell growth hexoses are known to support cell division while sucrose favors differentiation and maturation. This has lead to the proposal of an invertase/sucrose-synthase control theory, where high invertase activity promotes
initial cell growth through cell division and transition to growth driven by cell expansion is facilitated by an alteration in the hexose/sucrose ratio through a shift from invertase to sucrose-synthase paths of sucrose cleavage. (Wobus and Weber, 1999; Borisjuk et al., 2002; Borisjuk et al., 2003; Weschke et al., 2003; Koch, 2004). This further strengthens our conclusion that cell growth is the limiting factor for the overall corm growth at the higher temperature regime.

4.5. Sink regulation of leaf senescence

Even though it is tempting to assign the greater biomass accumulation at lower temperature simply to the prolonged leaf life, the collected data suggests that it is the events within the corm that limits the final size and, at least in part, dictates the leaf life span rather than the other way around. Thermoperiodicity, the change in mean temperature with the season, is believed to determine the onset of leaf senescence (Caldwell, 1969; Sawada et al., 1997). Constant low temperature delays foliage senescence, e.g. growth at 10°C cause the spring ephemeral, *Erythronium japonicum* to senesce one moth later than when grown at 20°C (Yoshie and Fukuda, 1994). Lapointe (2001) suggested that the timing of the foliar senescence is linked to the changes in sink demand and not a direct effect of any environmental cues, such as temperature. The inability to store further photoassimilates would result in an accumulation of carbohydrates in the leaves. High concentrations of sugars are known to repress photosynthesis and an increase in sugar content has been reported in leaves at the onset of senescence in both tobacco and *Arabidopsis* (Masclaux et al., 2000; Diaz et al., 2005), suggesting that sugar signaling play a role in the induction of senescence. More direct evidence came from *Arabidopsis* where growth in the presence of 2% glucose in combination with low nitrogen supply induced leaf yellowing (Pourtau et al., 2004; Wingler et al., 2004) and changes in gene expression typical of those seen during developmental leaf senescence (Pourtau et al., 2006). One characteristic of growth at low temperature is the accumulation of soluble sugars and an essential feature of cold acclimation is the ability to overcome the repression that this accumulation has on carbon metabolism. This is believed to in part be achieved by rendering the plant insensitive to sugar signals. We find it probable that this is part of the explanation of the prolonged epigeous growth period at low temperature in *C. vernus*.

![Fig. 7. Model of the influence of sink demand on leaf senescence, under two temperature regimes. For further details see Lapointe (2001).](image-url)
**Conclusions**

My thesis has extended the knowledge of the importance of reprogramming carbon metabolism and sustaining growth for achieving full cold acclimation in plants.

The simultaneous upregulation of the Calvin cycle and sucrose synthesis at low temperature without jeopardizing the positive effects on photosynthesis was found to be achieved by transcriptional control of the TPT. Carbon was instead found to be rerouted out of the chloroplast via breakdown of transient starch and MEX1.

The recovery of export, and thus the supply of growth materials to developing sinks, during cold acclimation were found to be facilitated through a combination of increased expression of sucrose transporters and an increase in vasculature.

The metabolome of cold stressed and cold grown plants were found to be substantially different, demonstrating that some of the alteration in e.g. carbohydrate statues generally associated with cold acclimation may actually be an effect of the stress rather than an effect of decreased growth temperature.

The thermal response of respiration and photosynthesis was established to be dynamic and prone to acclimation. The underlying biochemical adjustments and the acclimation potential were found to be remarkably similar between different functional groups and the R:A ratio did not always reach homeostasis, even in leaves developed at a new temperature regime.

The reduced perennial growth of *C. vernus* at higher temperature was not an effect of restricted carbohydrate supply, instead restriction in cell growth was suggested to be the limiting factor for the overall corm growth.
Köldacklimatisering-vikten av en funktionell metabolism

Säsongsrörade kyla och återkommande växlingar mellan frost och tå uppvisar en betydande begränsning odlingsbarheten hos en mängd kommersiellt viktiga grödor och träd. Köldknäppar under våren och hösten har dessutom en avservärt negativ effekt på storleken på nästkommande skörd genom att de medför skador på groddplantor eller ömtåliga organ såsom blommor och frukter. Cirka 10% av skördren i världen får förlorad pga av köldrelaterade skador, och globalt sett uppskattas frosten stå för förluster på över 14 miljarder US dollar årligen. Många växter kan dock rusta sig för den kommande frosten genom att köldacklimatisera, vilket skyddar cellerna och möjliggör bibehållandet av fysiologiska funktioner, såsom fotosyntesen, när temperaturen sjunker under noll. Under köldacklimatiseringen sker en rad förändringar som minskar de skadliga effekterna, en del av dessa har karakteriserats men det finns fortfarande stora luckor i vår kunskapen kring köldacklimatisering. Målet med min avhandling har varit att fylla igen några utav dessa.

En rad experiment har visat att ökad köldtolerans är sammankopplat med ett återupprättande av funktionell fotosyntesen, denna återhämtning är i sin tur knuten till en uppreglering av syntesen av sukros. Sukros utgör den viktigaste energikällan hos växten men är även en osmotisk förening med skyddande effekt.

Genom att hos transgena backtav plantor (Arabidopsis thaliana) påverka syntesen av sukros har vi kunnat påvisa att en ökad fixering av koldioxid, ökad inlagring av kol i form av sukros tillsammans med återupptagen export av sukros är nödvändig för att växten ska kunna återfå en fungerande metabolism vid låga temperaturer, och därigenom öka toleransen mot frost. Återupptagen export av sukros exporten, och därmed tillväxten av ny vävnad, är beroende av en uppreglering av genutryck för ett antal sukrotransportörer samt en ökning i mängen vaskulär vävnad.

Vidare har vi jämfört responser hos blad som köldstressats med blad från plantor som kultiverats vid konstant låga temperaturer, och därför inte utsatts för någon egentlig stress, för att utröna om det är några skillnader mellan en så kallad stressrespons med en renodlad köldrespons. Vi fann omfattande skillnader i metabolomet vilket indikerar att många utav de förändringar som har associerats till köldacklimatisering egentligen är en effekt av själva stressen.

Vi har även applicerat en del av den kunskap som erhållts genom studier av den traditionella modellorganismen backtav på andra mindre kända växter, av kontrasterande funktionella grupper (örter, gräs och vintergröna träd/buskar). Genom detta har vi visat att det finns stora likheter i förmågan att utveckla frosttolerans och de biokemiska förändringar som ligger bakom dessa mellan olika funktionella grupper.
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Eternitate adjungere, mot nya mål och upplevelser,


Amthor JS (2000) Direct effect of elevated CO$_2$ on nocturnal in situ leaf respiration in nine temperate deciduous tree species is small. Tree Physiology 20: 139-144


Arnone JA, Körner C (1997) Temperature adaptation and acclimation potential of leaf dark respiration in two species of Ranunculus from warm and cold habitats. Arctic and Alpine Research 29: 122-125


Beebe DS, S, Blennow A (2005) A

Baxter urner J , Ro lfe SA, Quick WP (2003) Elevated sucrose-


- 51 -


Fowler S, Thomasow MF (2002) Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 14: 1675-1690


translocator on photosynthetic metabolism in transgenic potato plants. Planta 193: 174-180


Hurry VM, Gardeström P, Öquist G (1993) Reduced sensitivity to photoinhibition following frost-hardening of winter rye is due to increased phosphate availability. Planta 190: 484-490


Transcription Factors Involved in Cold-responsive Gene Expression in Transgenic Rice. Plant Cell Physiol. 47: 141-153


Parker J (1962) Relationships among cold hardness, water-soluble protein, anthocyanins, & free sugars in {lHedera helix} L. Plant Physiology 37: 809-813
Philpott J (1956) Blade tissue organization of foliage leaves of some Carolina shrub-bog species as compared with their Appalachian mountain affinities. Botanical Gazette 118: 88-105
Purvis AC (1997) Role of the alternative oxidase in limiting superoxide production by plant mitochondria. Physiologia Plantarum 100: 165-170


lipid composition. Proceedings of the National Academy of Science USA 85: 9026-9030


Urrutia ME, Duman JG, Knight CA (1992) Plant thermal hysteresis proteins. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1121: 199-206
Vanlerberghe GC, McIntosh L (1992) Lower growth temperature increases alternative pathway capacity and alternative oxidase protein in tobacco. Plant Physiology 100: 115-119


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