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PHOSPHO*ENOL*PYRUVATE CARBOXYLASE AND COLD ACCLIMATION OF ALFALFA

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July,1996

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Master of Science

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ABSTRACT

Phospho*enol*pyruvate carboxylase (PEPC) was examined during cold acclimation of seedlings of the freezing-tolerant cultivar (*Medicago sativa* ssp *falcata* cv Anik) and the relatively freezing-sensitive cultivar (*Medicago sativa* cv Trek) of alfalfa. With four days of cold acclimation, PEPC activity increased to 3.5-fold and 2-fold the control levels in Anik and Trek, respectively. This was associated with an increase in the level of a 110 kD PEPC protein and a decrease in the amount of a 120 kD PEPC polypeptide in both cultivars. The role of reversible phosphorylation in regulating PEPC activity was demonstrated by *in vitro* phosphorylation and dephosphorylation, which caused partial activation and deactivation of PEPC, respectively. *In vivo* phosphorylation experiments revealed that the 110 kD PEPC subunit is phosphorylated on serine residue(s) during cold acclimation in Anik but not in Trek. Increased PEPC activity could account for the 70% increase in the non-autotrophic or dark fixation of carbon observed in cold acclimated Anik seedlings. A possible role for dark carbon fixation in the cold-induced development of freezing tolerance is through the production of NADPH. Such a source of reducing power may be required for the repair of cold-induced damage and restoration of normal cellular functions.

RÉSUMÉ

Phosphoenolpyruvate carboxylase (PEPC) fut examiné pendant l'acclimatation au froid d'un cultivar tolérants à la congélation (*Medicago sativa* ssp *falcata* cv Anik) et d'un autre relativement sensibles à la congélation (*Medicago sativa* cv Trek) d'alfalfa. Après quatre journées d'acclimatation au froid, l'activité de PEPC a augmenté à un nivau 3.5 et 2.5 fois plus élevé que les nivaux des controles d'Anik et de Trek, respectivement. Ceci fut associé à une augmentation dans la quantity d'une protéine PEPC de 110 kD et d'une diminution de celle d'un polypeptide de 120 kD dans les deux cultivars. Le rôle de la phosphorylation réversible dans le contrôle de l'activité de PEPC fut démontré par la phosphorylation et la déphosphorylation in vitro résultant dans l'activation et la inactivation partielle de PEPC, respectivement. Les expériences de phosphorylation in vitro ont révélé que la protéine PEPC de 110 kD est phosphorylée sur un résidu sérine pendant l'acclimatation au froid dans Anik mais pas dans Trek. L'augmentation de l'activité de PEPC peut expliquer entierement l'augmentation de 70% dans la fixation du carbone non-autotrophique ou dans l'obscurité observée dans des pousses d'Anik acclimatées au froid. Un rôle possible de la fixation du carbone à la'obscurité pendant dévelo[[ement de la tolérance à la congélation serait par la production de NADPH. Une telle source de reducteur peut être requise pour la réparation de dommages induits par le froid et pour la restoration des fonctions normales.

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PREFACE

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made

more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

This thesis is being submitted in the manuscript-based format. It consists of a literature review, followed by the manuscript submitted to "Plant Physiology", entitled "Modulation of Phospho*enol*pyruvate Carboxylase Activity during Cold Acclimation of Alfalfa" and co-authored by Scott A. Frank, Antonio F. Monroy and Rajinder S. Dhindsa. A list of literature cited for both components of the thesis is combined at the end.

Scott A. Frank is responsible for the execution of all experiments and for much of the writing of the manuscript.

Antonio F. Monroy contributed to the paper by experimental planning and analysis and in editing of the manuscript.

Finally, Rajinder S. Dhindsa generated the experimental direction, supervised and financed the research, and provided suggestions for the improvement of the manuscript.

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INTRODUCTION AND LITERATURE REVIEW

Plants undergo normal growth and metabolism only when conditions are optimal. However, many areas of the earth typically exhibit one or more factors which are in excess or deficit, imparting stress to both wild and cultivated plants. These stresses result in reduced growth, poor quality of plant nutrition, and even death, resulting in agricultural losses around the world.

Unlike animals, plant species cannot actively avoid stressful conditions by virtue of mobility, but rather deal with stress by two mechanisms: either avoidance or tolerance. Avoidance refers to the ability of a plant to maintain normal metabolism even though the external environment is heavily stressful. For example, some plants grow normally in soils containing toxic levels of aluminum with the aid of exclusion mechanisms which prevent the metal from being taken up into the plant (Delhaize and Ryan, 1995). Stress tolerance describes the ability of a plant to develop normally under internal as well as external stress conditions. In the case of aluminum-tolerant plants, internal mechanisms exist which allow the plant to function normally under high concentrations of internal aluminum (Delhaize and Ryan, 1995).

There are many different types of stresses which restrict agricultural output including drought and salt stress, metal toxicity, mineral deficiencies and anaerobic stress due to flooding. Of particular concern in northern climates is low-temperature stress which causes reductions in seed germination, plant growth and reproductive ability (Andrews, 1987). In Canada, alfalfa is a major agronomic crop which is used cattle feed

by dairy industries. There are many varieties of this perennial plant, some of which are able to survive freezing, and are therefore, capable of overwintering, and others that are not. Examination of the similarities and differences among these varieties serves as a useful model for understanding stress tolerance and adaptive mechanisms, in addition to being potentially useful in the development of improved agricultural crops.

PLANTS AND COLD STRESS

As winter approaches and the temperature declines, plants experience low-temperature stress. Cold stress can be due to chilling, which is observed when plants located in tropical or subtropical climates are subject to non-freezing temperatures, or due to freezing which occurs when plants in northern or temperate climates experience temperatures below 0 °C (Levitt, 1980). Both of these stresses, of which we will focus on freezing, may be temporary or permanent, and cause direct and indirect injury to plants, resulting in reduced plant viability.

Low-temperature damage includes a decrease in membrane fluidity resulting in membrane deformation and altered permeability (Levitt, 1980). In addition, ice crystal penetration inside the cell can rupture intracellular organelles including mitochondria (Perras and Sarhan, 1984), causing decompartmentation of the cell and loss of organellar functions. Indirect injury occurs as a result of ice crystallization in the extracellular space. As solutes accumulate with ice crystallizing in the intercellular space, the cell experiences dehydration stress as water moves osmotically out of the cell (Levitt, 1980).

It is therefore, thought that drought is a significant component of freezing injury and that tolerance to freezing stress implies drought-tolerance (Lin et al., 1990; Levitt, 1980). The low-water content of the cell can result in oxidative stress which can alter redox balance (Dhindsa, 1991; Vanlerberghe et al., 1989; Levitt, 1980) causing significant negative consequences to the plant including reduced protein synthesis (Mohapatra et al., 1987) via the generation of harmful oxidative radicals (Ingram and Bartels, 1996; Elstner, 1982).

In competent genotypes, this damage results in the induction of metabolic changes that may play a role in responding to the stress and allowing the plant to survive freezing and to overwinter. Adaptive changes to the low-temperature stress include specific gene expression, increased membrane fluidity, changes in the levels and activities of enzyme isoforms, the accumulation of soluble protein and intracellular osmoprotectants, and the appearance of antifreeze proteins (Guy, 1990; Levitt, 1980). The magnitude of these changes depends upon the degree of the stress, and can have significant effects on the chance of survival.

As mentioned above, not all plant species are capable of surviving freezing-stress (ie. it is genotype-dependent), nor do they all necessarily employ the same mechanisms of withstanding sub-zero temperatures. There is a continuum of tolerance ranging from freezing-tolerant plant species to those which are irreversibly damaged by exposure to low-temperature (ie. chilling-sensitive).

PLANTS AND COLD ACCLIMATION

The ability to withstand freezing temperatures and to overwinter is common to many Canadian plants including agronomically important crops such as alfalfa. This freezing tolerance can be induced by exposure to low, but non-freezing temperatures (cold acclimation). Cold acclimation, or cold hardening, occurs naturally in the fall and results in the accumulation of the plant stress hormone abscisic acid (ABA), and the inhibition of growth (Guy, 1990; Levitt, 1980).

The ability to cold acclimate and attain freezing-tolerance is a genotype-dependent process that may be aided by a number of factors. Freezing-tolerance increases with greater length of cold acclimation and with lower acclimating temperature. A change in photoperiod may also aid in priming the plant for cold acclimation as decreasing sunlight during the fall signals the oncoming winter. Younger organs tend to acclimate better than older ones and aerial parts of the plant also exhibit a greater ability to cold acclimate than organs which are not exposed during winter. As with most plant processes, the ability to cold acclimate also depends on adequate nutrition (Levitt, 1980; Weiser, 1970).

Cold acclimation can be thought to be comprised of two separate functions: firstly, as the adjustment of metabolism and cellular processes to low temperature which occurs in chilling-tolerant, but not in chilling-sensitive plants; and secondly, as the induction of freezing tolerance, which occurs only in freezing-tolerant plants (Guy, 1990). Freezing tolerance is assessed by measuring the percent survival of cold-

acclimated plants at a given temperature or determining the temperature at which half of the acclimated plants survive (LT_{50}). The LT_{50} varies greatly between plant species allowing annual and biennial plants to tolerate temperatures ranging from -10 to -30°C and perennials to withstand temperatures as low as -60°C (Weiser, 1970).

Development of freezing tolerance is a process which is inducible by exposure to low-temperature as well as by other methods. Exogenous treatment of a plant with ABA (Chen and Gusta, 1983; Chen et al., 1983) or dessicating conditions, (Siminovitch and Cloutier, 1982) which are known to result in the accumulation of ABA (Wright, 1977), both induce plant tolerance to subsequent freezing temperatures. This suggests that the responses to dessication and cold acclimation may share common adaptive mechanisms as has been proposed by Lin et al. (1990) and Levitt (1980).

In comparison to heat shock and the development of thermotolerance, freezing tolerance is a slow process, taking up to months depending on the plant species, whereas deacclimation and loss of freezing tolerance are more rapid processes. Some perennial plants are able to acclimate and deacclimate very rapidly in order to adapt to temperature fluctuations during winter (Guy, 1990).

The capacity to cold acclimate is a complex, polygenic trait (Hummel et al., 1982; Norell et al., 1986). It is interesting that under mild stress, cold acclimation is a dominant trait, whereas it is recessive under more severe conditions (Sutka and Veisz, 1988), supporting the idea that different genes effect tolerance at different levels of stress (Gullord et al., 1975). Several genes have been shown to be specifically expressed with cold acclimation and are well-characterized (Monroy et al., 1993b; Wolfraim et al.,

1993; Wolfraim and Dhindsa, 1993; Houde et al., 1992; Mohapatra et al., 1989). Although the appearance of some proteins and disappearance of others may not be directly related to freezing tolerance, the findings that some genes co-segregate with freezing tolerance (Thomashow, 1990), and that cold-acclimation specific (*cas*) genes are induced to a greater degree in freezing-tolerant than in sensitive cultivars (Houde et al., 1992; Mohapatra et al., 1989), strongly suggest that the proteins encoded by certain genes may play important roles in the cold-induced development of freezing tolerance.

PROTEINS AND COLD ACCLIMATION

It has been shown that protein metabolism plays a major role in cold acclimation and the development of freezing tolerance (Guy, 1990). Siminovitch and Briggs (1949) first noted the correlation between soluble protein accumulation and freezing tolerance during cold acclimation of black locust trees. The increased protein content may be due to increased synthesis and stability of proteins and/or reduced degradation and turnover. In addition to increased overall soluble protein content with cold acclimation as a general stress response in many plant species, the levels of many individual proteins increase or decrease. Results from SDS-PAGE, in vivo radiolabeling and experiments with cycloheximide have demonstrated that changes in protein patterns occur with cold acclimation, and that many of these changes are due to novel protein synthesis (see Guy,

1990).

Comparative studies of proteins of non-acclimated and cold acclimated plants have identified proteins that are correlated with freezing-tolerance. In spinach, time-course studies have demonstrated the synthesis and appearance of high-molecular weight proteins during the induction of freezing tolerance and their disappearance during deacclimation (loss of freezing tolerance) (Guy and Haskell, 1987), suggesting a central role in freezing tolerance. These proteins may aid in stabilizing key proteins, be structurally important, or represent cold-stable isoforms of enzymes catalyzing housekeeping or cold-specific metabolic processes.

The appearance of many new proteins upon exposure to low temperature is due to both altered gene expression, as proposed by Weiser (1970), and upregulation of many genes (Guy, 1990). The ability to synthesize and maintain the concentration of housekeeping proteins at low temperature is greater in cold acclimated plants than in non-acclimated plants due to increased rRNA and protein-synthesis capacity, consistent with the need to sustain basal metabolism at reduced temperature (Guy, 1990). Other candidates for induction or enhanced expression are antifreeze proteins, which restrict ice crystal growth from the protoplasm (Griffith et al., 1992), and cold-stable enzymes involved in the synthesis of cryoprotectants, such as sorbitol, proline, glycinebetaine and sucrose, which is required for the development of freezing tolerance (Steponkus and Lanphear, 1968). Antioxidants, such as ascorbate peroxidase, glutathione reductase and superoxide dismutase, which function in the removal of toxins, have also been shown to be upregulated in response to oxidative stress (Ingram and Bartels, 1996; McKersie

et al., 1993; Dhindsa, 1991; Elstner, 1982). Proteins involved in the production of lipids for membrane biosynthesis and restructuring, including the activation of desaturases to increase membrane fluidity, are also important candidates for enhanced synthesis during cold acclimation because of the extensive damage incurred to biological membranes during freezing (Guy, 1990; Levitt, 1980).

Protein regulation by reversible phosphorylation

It is also possible that post-translational modifications to proteins could enhance stability or alter activities with concomitant effects to cellular metabolism. Phosphorylation is an important universal mechanism for regulating biological functions in response to external stimuli or intracellular signals (Smith and Walker, 1996). Changes in the relative phosphorylation levels of target proteins are brought about by the opposing actions of protein kinases and protein phosphatases. The kinases are responsible for the addition of phosphate groups to proteins (phosphorylation) whereas phosphatases are responsible for the reverse process (dephosphorylation). The actions of these modifying enzymes shifts the equilibrium to the more or less phosphorylated state and directly effects the activities of target proteins, which can also affect downstream processes via signal transduction cascades. In eukaryotes, 97% of protein phosphorylation occurs at serine and threonine residues, although tyrosyl-phosphorylation also plays an important role in signal transduction (Smith and Walker, 1996).

Changes in phosphoprotein profiles occur in response to a number of internal and

external stimuli including transitional stages of development (Nakamura et al., 1993; Raghothama et al., 1985), the important plant stress hormone ABA (Koontz and Choi, 1993) and environmental cues (Bursa et al., 1994; Wu et al., 1992), although the mechanisms inducing plant responses have yet to be established.

Although there is a significant amount of research demonstrating increased protein content with cold acclimation (see Guy, 1990), the effect of low-temperature stress on protein phosphorylation is in its infancy. Thylakoid proteins have been shown to be affected by chilling in many species (Val and Baker, 1989; Moll et al., 1987), and cold has been shown to affect the phosphorylation status of the light-harvesting complex, resulting in changes in the organization of the thylakoid membrane (Carlberg et al., 1992), and presumably affecting photosynthesis. Research in this laboratory on the effects of cold acclimation on protein phosphorylation has clearly demonstrated changes in phosphoprotein profiles during cold acclimation (Monroy et al., 1993a). Some of these changes have been shown to be dependent on calcium and protein synthesis as well as require the action of protein kinases (Monroy et al., 1993a) and protein phosphatases (Monroy et al., 1996).

Stone and Walker (1995) have recently reviewed the different types of plant protein kinases and their role in signal transduction, many of which are inducible by stress. Urao et al. (1994) reported that two genes encoding protein kinases were induced by drought and salt stress, an ABA-inducible kinase has been isolated from wheat (Anderberg and Walker-Simmons, 1992), a pathogenesis-related kinase has been cloned from tomato (Martin et al., 1993) and a cold-induced calcium-dependent protein

kinase(CDPK) has been identified in alfalfa (Monroy and Dhindsa, 1995). Although, the phosphorylation of proteins by kinases has been demonstrated in many systems, the signaling pathways leading to altered gene expression and physiological responses have not yet been elucidated.

It is only recently that the importance of research on protein dephosphorylation by protein phosphatases has been recognized, and as usual, plant studies lag far behind animal research. However, homologues of the mammalian protein phosphatase classes 1, 2A and 2C have been found in plant species, in addition to unrelated phophatases, and the role of dephosphorylation in regulating enzyme activities and cellular processes is becoming clear (Monroy et al., 1996; Smith and Walker, 1996).

Although recent attention has been given to the modifying kinases and phosphatases, most research remains focused on the effect of phosphorylation status on protein function. Enzymes involved in many different cellular processes have been found that exhibit altered activities when phosphorylated (see Ranjeva and Boudet, 1987). Phosphorylation is involved in regulating the cell cycle, gene expression, and is an almost universal requirement for the activation of protein kinases themselves (Stone and Walker, 1995). Some plant enzymes that have been shown to be regulated by reversible phosphorylation include pyruvate dehydrogenase, pyruvate Pi dikinase and the important carbon-metabolism enzyme, phosphoenolpyruvate carboxylase (Ranjeva and Boudet, 1987).

PHOSPHO*ENOL*PYRUVATE CARBOXYLASE

Phospho*enol*pyruvate carboxylase (PEPC) (EC 4.1.1.31) is a well-characterized cytosolic enzyme that occurs ubiquitously in plants, animals and bacteria (for a recent review of PEPC in plants, see Chollet et al., 1996). This enzyme, which is a homotetramer comprised of approximately 110 kD subunits, catalyzes the irreversible ß-carboxylation of phosphoenolpyruvate (PEP)in the presence of metal ions and HCO_3^-, to generate oxaloacetate and inorganic phosphate. In addition to the well-known role of this enzyme as the primary carbon-fixing enzyme in the leaves of plants operating under C_4 photosynthesis (C_4 plants) or Crassulacean acid metabolism (CAM plants), PEPC has many other important functions. These include the replenishment of tricarboxylic acid cycle intermediates providing carbon skeletons for nitrogen assimilation and amino acid biosynthesis, maintenance of redox balance, recapture of respiratory CO_2 and transfer of reducing power from NADH to generate NADPH by the non-autotrophic or dark fixation of carbon. Other specialized roles include the provision of C_4-dicarboxylate substrates to nitrogen-fixing bacteria in the root nodules of leguminous plants, and production of malate as an osmotic solute for the regulation of stomatal aperture and generation of turgor-driven growth (Dhindsa et al., 1975). Associated with a variety of functions and intracellular localizations of PEPC, there are different isoforms which are encoded by multigene families (Lepiniec et al., 1994).

In the leaves of C_4 or CAM plants, PEPC is the initial carboxylating enzyme that fixes atmospheric CO_2 into dicarboxylates. The enzyme is active during the day in C_4

and during the night in CAM plants. During the dark and light for C4 and CAM plants, respectively, the enzyme is less active and the CO_2 is released from the dicarboxylic acids to serve as substrate for the Calvin cycle. In the case of C_4 PEPC, it has been found that light reversibly induces a two- to three-fold increase in catalytic activity and decrease in sensitivity to the inhibitor L-malate, when assayed at suboptimal, but physiological levels of pH and PEP (reviewed by Chollet et al., 1996 and Jiao and Chollet, 1991). Similarly, CAM plants exhibit significant day-night differences, PEPC activity being higher in the night so that the stoma can remain closed during the day to conserve water. Although there are light-induced changes in the levels of opposing metabolite effectors (positive regulator glucose-6-phosphate and negative regulator L-malate), the major mechanism of regulating PEPC activity has been found to be reversible phosphorylation. This post-translational modification occurs specifically at a serine residue near the N-terminus of the protein. This covalent modification occurs at Ser-8 in *Sorghum*, at Ser-15 in maize and at Ser-11 in the facultative CAM plant *Mesembryanthemum crystallinum* (Chollet et al., 1996; Lepiniec et al., 1994). Moreover, it is now clearly established that this seryl-phosphorylation of PEPC directly results in increased catalytic activity and decreased sensitivity to inhibition by malate, both in vitro and in vivo (see review by Chollet et al., 1996 and references therein).

Further research on PEPC has revealed that the non-photosynthetic isoforms of the enzyme are regulated by reversible phosphorylation as well. Regulatory seryl-phosphorylation of PEPC plays an important role in the coordination of carbon-nitrogen metabolism in the leaves of C_3 plants (Duff and Chollet, 1995; Van Quy et al., 1991).

In addition, the PEPC-mediated generation of dicarboxylic acids to supply respiratory substrates and carbon skeletons for amino acid biosynthesis to nitrogen-fixing bacteria in legume nodules is regulated by reversible phosphorylation at serine residue(s) (Zhang et al., 1995; Schuller and Werner, 1993). Phosphorylation of PEPC has been implicated in the regulation of stomatal aperture in *Vicia faba* guard cells (Zhang et al., 1994).

There has been a great deal of research on the signal transduction pathway leading to the activation of PEPC. Earlier reports indicated that the activity of PEPC was dependent on the oligomeric status of the enzyme (tetrameric form more active than dimeric form) in both C_4 and CAM species (Wu and Wedding, 1985 and 1987). However, the findings that the light and dark forms of PEPC from C_4 and CAM plants exist as tetramers, yet exhibit their characteristic differences in activity and sensitivity to inhibition, effectively argues against this aggregation hypothesis (see Jiao and Chollet, 1991).

Researchers have found that upstream events are required for the phosphorylation and therefore, activation of PEPC. These include roles for: cytosolic alkalinization and increased cytosolic calcium (Giglioli-Guivarc'h et al., 1996; Pierre et al., 1992), calcium-dependent protein kinases (Giglioli-Guivarc'h et al., 1996), cross-talk between the photosynthetic cell types of the C_4 leaf (Giglioli-Guivarc'h et al., 1996; Jiao and Chollet, 1992), and protein synthesis (Giglioli-Guivarc'h et al., 1996; Jiao and Chollet, 1992; Pierre et al., 1992; Jiao et al., 1991b).

More recently, attention has turned to the PEPC-modifying protein kinases and phosphatases. In all sources studied to date, the PEPC kinase has been found to be a

calcium-independent protein-serine/threonine kinase that is tightly regulated (Chollet et al., 1996). Although both calcium-dependent and -independent kinases are capable of phosphorylating PEPC in vitro and consequently altering its activity and malate sensitivity (Li and Chollet, 1993), the calcium-independent kinase is likely the in vivo regulating enzyme, because of its light-dependency and sensitivity to inhibitors of protein synthesis and photosynthesis (Li and Chollet, 1993). As mentioned above, these factors have been implicated in the regulation of PEPC, indicating that activation of PEPC requires the activated synthesis of the modifying kinase itself.

There is much less work on the dephosphorylation event that inactivates PEPC, although it has been found that it involves a type 2A protein phosphatase (Jiao et al., 1991a; Echevarria et al., 1990). However, this enzyme is likely to be less important in the regulation of PEPC since its activity is fairly constant during light-dark or day-night transitions (Chollet et al., 1996) and hence, most attention is directed toward the regulation of PEPC by its serine-threonine kinase.

The diversity of function of the PEPC isoforms is consistent with the finding of small multigene families in many plant species (Lepiniec et al., 1993 and 1994). In the common ice plant, at least two distinct genes for PEPC encode the C_3-like and CAM-specific isoforms, the expression of the latter is enhanced as the plant switches from C_3 to CAM in response to salt stress (Cushman et al., 1989). *Sorghum* possesses three different genes encoding the housekeeping, root and C_4-photosynthetic isoforms (Lepiniec et al., 1993), and maize contains at least five genes for PEPC. Many other

species contain PEPC multigene families including tobacco, rice, wheat and alfalfa (Lepiniec et al., 1994; Pathirana et al., 1992).

In all amino acid sequences of plant PEPC reported to date, several highly conserved residues and motifs are found (Lepiniec et al., 1994). These domains are likely to be important for enzyme activity, and probably either represent components of the active site or are involved in regulation of the enzyme. As mentioned above, the major mechanism of regulating PEPC is by reversible phosphorylation. Consistent with this, one of the most conserved regions in all PEPC's sequenced to date is the seryl-phosphorylation site near the N-terminus of the protein (E/DR/KxxSIDAQL/MR). It is this domain that confers the ability to regulate PEPC activity as has been confirmed by the inability to phosphorylate and therefore alter the properties of phosphorylation-site mutants of *Sorghum* (Duff et al., 1995).

Cold acclimation, phospho*enol*pyruvate carboxylase and dark carbon fixation: Basis of the thesis project

It is well-known that freezing stress causes great perturbations to the plant. Damage to the plasma membrane and internal organelles (Levitt, 1980), protein-synthesizing machinery (Mohapatra et al., 1987) and alteration of redox balance due to dehydration (Ingram and Bartels, 1996; McKersie et al., 1993; Clare et al., 1984; Dhindsa and Matowe, 1981) are all consequences of cold-exposure. A source of reducing power which can be used to repair biological components damaged by the

freezing stress may be important in imparting freezing-tolerance to the plant. A major mechanism of generating reductant is the production of NADPH by the non-autotrophic or dark fixation of carbon. This pathway, which is catalyzed by PEPC (Schuller et al., 1990b), coupled with transhydrogenation from NADH to $NADP^+$ (Ting and Dugger, 1965a) is well-characterized in both plants and animals (Ting, 1971). Moreover the role of regulatory phosphorylation of PEPC on dark carbon fixation during CAM has been demonstrated (Carter et al., 1995). Dark carbon fixation has been implicated in the ability to recover from desiccation in mosses (Dhindsa, 1985) and in restoration of redox balance during anaerobiosis (Vanlerberghe et al., 1989). Furthermore, NADPH ameliorates oxidative damage to protein synthesis (Dhindsa, 1987), and is required for the reductive biosynthesis of membrane components (Stumpf, 1980). We therefore, decided to investigate whether there was a change in the level of dark carbon fixation that occurs with cold acclimation of alfalfa, and whether PEPC was responsible for some, if not all, of any such alteration.

PROJECT AIMS

Alfalfa is a major agronomic crop in Canada producing hay and forage for the cattle and dairy industries of North America. Production of this over-wintering crop accounts for 300 million dollars in Quebec and 1 billion dollars in Canada as a whole. Once planted, this crop can be harvested for 3-4 years although a small percentage of plants die each year from freezing. It is thought that an increase in the ability of plants

to survive the freeze-thaw process will result in increased agricultural productivity, particularly in light of the fact that the high-yielding cultivars are freezing-sensitive (ie. Trek) whereas the lower-yielding cultivars are relatively freezing-tolerant (ie. Anik). The wide range of freezing-tolerance of the various alfalfa species and cultivars, coupled with the agronomic importance of this plant and the availability of cold-acclimation specific (*cas*) genes as markers for cold acclimation (Wolfraim and Dhindsa, 1993; Wolfraim et al., 1993; Monroy et al., 1993b; Mohapatra et al., 1989), make it a good system for the study of cold acclimation and freezing tolerance.

The damaging effects of freezing to plants are well-characterized (Levitt, 1980). It is logical that a source of reducing power to repair damage and restore metabolic functions may be of importance towards imparting freezing tolerance to the plant. The generation of NADPH by the non-autotrophic or dark fixation of carbon may be the source of such reductant. We, therefore, decided to examine whether any change in the level of dark carbon fixation occurs with cold acclimation, and we were particularly interested in the principle enzyme catalyzing this process, PEPC. By examining the mRNA, protein and activity levels, as well as the phosphorylation status of PEPC during cold acclimation, we hoped to determine whether PEPC could be responsible for any alterations in the level of dark carbon fixation. We also aimed to identify the mechanism by which the enzyme is regulated in this process.

This investigation represents novel research in the field of cellular metabolism in response to cold acclimation and the development of freezing tolerance. Although any findings are purely correlative (i.e. we do not know if increased dark carbon fixation

and/or PEPC activity are directly involved with increased freezing tolerance), the identification of any differences between freezing-tolerant and -sensitive cultivars that arise due to cold acclimation, would suggest at least a partial role, and may also shed light on different plant responses to stress. Furthermore, these findings may reveal any cold-induced components which are absent or defective in the sensitive plants, and may therefore be useful to the commercial development of alfalfa strains with improved agricultural productivity.

Modulation of Phospho*enol*pyruvate Carboxylase Activity during Cold Acclimation of Alfalfa[1]

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ABSTRACT

Phospho*enol*pyruvate carboxylase (PEPC) activity and levels were examined during cold acclimation of seedlings of the freezing-tolerant cultivar (*Medicago sativa* ssp *falcata* cv Anik) and the relatively freezing-sensitive cultivar (*Medicago sativa* cv Trek) of alfalfa. PEPC activity increased 250 % and 100 % with cold acclimation in Anik and Trek, respectively. This was associated with an increase in the level of a 110 kD protein and a decrease in the amount of a 120 kD polypeptide, both of which immunoreacted with an anti-PEPC antibody. Incubation of soluble protein extracts with ATP increased PEPC activity by about 30 % while incubation with alkaline phosphatase decreased PEPC activity by about 70 % in Anik seedlings. Immunoprecipitation of proteins from extracts with antiphosphoserine antibody followed by immunoblotting of the precipitated proteins with anti-PEPC antibody revealed the 110 kD polypeptide only in cold acclimated Anik seedlings. These findings suggest that the 110 kD PEPC subunit is phosphorylated on serine residue(s) during cold acclimation in Anik but not in Trek. Increased PEPC activity could account for the 70% increase in non-autotrophic carbon fixation observed in cold acclimated Anik seedlings. A possible role for dark carbon fixation in the cold-induced development of freezing tolerance is discussed.

INTRODUCTION

Exposure to low, but nonfreezing temperatures (cold acclimation) confers on many plants tolerance to subsequent freezing temperatures (Levitt, 1980; Guy, 1990). During cold acclimation, many metabolic changes occur including specific gene expression and protein synthesis (Guy, 1990; Thomashow, 1990). During the initial stages of cold acclimation, a transient damage to various cellular constituents is observed. For example, ATP levels decline (Perras and Sarhan, 1984) indicating mitochondrial dysfunction, solute leakage is manifested indicating membrane dysfunction (see Levitt, 1980), and a reduced rate of protein synthesis is observed even on return to normal temperature (Mohapatra et al., 1987) indicating an impairment of the protein-synthesizing apparatus. As cold acclimation proceeds, these damages are reversed and the plant develops increased freezing tolerance. There is considerable evidence that oxidative damage occurs on exposure to low temperature thereby altering the cellular redox state (Levitt, 1980). Availability of NADPH as a reductant is expected to be of importance during damage repair and restoration of cellular functions. An important way of producing NADPH is through dark fixation of carbon catalyzed by phospho*enol*pyruvate carboxylase (PEPC) coupled with transhydrogenation from NADH to $NADP^+$ (Ting and Dugger, 1965a). It has been proposed (Vanlerberghe et al., 1989) that provision of reducing power by dark or non-autotrophic fixation of carbon may contribute to overall redox balance in cells during anaerobic conditions. Furthermore, ability to recover from complete desiccation in mosses has been correlated

with an ability to resume dark carbon fixation (Dhindsa, 1985) and the ameliorative effects of NADPH on oxidation damage to protein synthesis have been demonstrated (Dhindsa, 1987). Therefore, we considered a possible role of PEPC during cold acclimation worthy of investigation.

PEPC (EC 4.1.1.31) is a ubiquitous enzyme that catalyzes the irreversible conversion of PEP and HCO_3^- to oxaloacetate and Pi (for a recent review, see Chollet et al., 1996). This enzyme, which is a homotetramer comprised of approximately 110 kD subunits, accounts for 0.5-2% of the soluble protein in alfalfa (Deroche and Carrayol, 1988; Vance et al., 1994). In leaves of C_4 and CAM plants, PEPC is the initial carboxylating enzyme that fixes atmospheric CO_2 into C_4 dicarboxylates. In C_3 leaves and N-fixing nodules of leguminous plants, PEPC plays an anapleurotic role in the provision of C skeletons for N assimilation into amino acids (Schuller and Werner, 1993; Duff and Chollet, 1995). PEPC has been shown to be post-translationally regulated by reversible phosphorylation at a serine residue. The phosphorylated enzyme is more catalytically active, and also more sensitive to activation by glucose-6-P and less sensitive to inhibition by L-malate (reviewed by Jiao and Chollet, 1991). Leaf PEPC is phosphorylated in the day in C_4 plants and in the night in CAM plants when the enzyme is active in the primary fixation of CO_2 in photosynthetic reactions. The enzyme is dephosphorylated during the night in C_4 and during the day in CAM plants (i.e. when it is not needed). The C_3-leaf and nodule PEPC isoforms are regulated in the same manner (Duff and Chollet, 1995; Zhang et al., 1995).

In this paper, we examined the activity, protein levels and phosphorylation status of PEPC during cold acclimation in two alfalfa cultivars differing in freezing tolerance. We show that PEPC activity increased with cold acclimation in both the freezing-tolerant cultivar Anik and the relatively freezing-sensitive cultivar Trek, although to a greater degree in Anik. This difference between cultivars was not due to variations in the PEPC transcript or protein levels, but rather due to regulatory seryl phosphorylation of the 110 kD PEPC subunit in Anik but not in Trek. The increased PEPC activity could account for the enhanced dark fixation of carbon that occurred with cold acclimation in Anik seedlings. A possible role for dark carbon fixation in the cold-induced development of freezing tolerance is discussed.

MATERIALS AND METHODS

Plant Material

Seeds of 2 alfalfa cultivars *(Medicago sativa* ssp *falcata* cv Anik, and *Medicago sativa* cv Trek) were surface sterilized in 5% (w/v) sodium hypochlorite and germinated in Murashige-Skoog medium (Sigma, St. Louis), pH 5.7 containing 1% sucrose. Seedlings were grown in liquid-culture at a PPFD of 200 μmol photons $m^{-2} s^{-1}$ under a 16-h photoperiod with shaking at 24° C. After 10 d, some of the seedlings were maintained as the non-acclimated sample while the remaining seedlings were transferred to 4° C, under otherwise identical conditions, for cold acclimation.

Protein Extraction

Seedlings were sampled at different times during cold acclimation starting with zero time as non-acclimated control. Samples were immediately frozen in liquid N_2 and stored at -80° C until further use. All steps in the extraction of protein were carried out at 4° C. The frozen seedlings (1-2 g fresh weight) were ground in liquid N_2 in a chilled mortar. The resultant powder was homogenized in pre-chilled extraction buffer containing 100 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 20% (v/v) glycerol, 1 mM EDTA, 2 mM 4-(2-Aminoethyl)-benzenesulfonyl fluoride (AEBSF) and 10 μM leupeptin (Boehringer Mannheim, Laval) and 10 mM ß-mercaptoethanol in a 1:4 (w/v) tissue:buffer ratio. The extract was filtered through Miracloth (Calbiochem, San Diego) and centrifuged at 15000 x g for 5 min. The supernatant served as a source of soluble protein for enzyme assays, determination of soluble-protein concentration, and PEPC immunoblotting and immunoprecipitation, and was used either immediately or aliquoted for storage at -80° C.

Enzyme Assays

PEPC activity was measured under suboptimal but physiologically relevant conditions (Schuller et al., 1990a; Duff et al., 1995), at 25° C using a radiometric assay. The 600 μL reaction mixture contained 50 mM Bicine, pH 7.5, 1 mM $MgCl_2$, 0.1 mM NADH, 0.4 μCi $NaH^{14}CO_3$ (56 mCi/mmol) and 0.25 mM PEP. All reactions were initiated with an aliquot of the protein extract and allowed to proceed for 5 min. The reaction was terminated with 600 μL of 10% TCA and unreacted ^{14}C was diluted with

the addition of 8 mM $NaHCO_3$. The mixture was centrifuged at 15000 x g for 5 min and an aliquot of the supernatant was removed for measurement of ^{14}C incorporation into acid-stable products by liquid scintillation counting. This was taken as the PEPC activity, and is expressed as cpm per μg soluble protein per min. For the optimal PEPC assay, the conditions were the same as above except that the buffer was adjusted to pH 8, and 2.5 mM PEP was used.

Malate dehydrogenase and malic enzyme activities were assayed spectrophotometrically at 25° C by measuring the change in absorbance at 340 nm. An aliquot of the soluble protein extract was added to the 3-mL reaction mixtures prior to initiation of reactions with the addition of oxaloacetate or L-malate for the malate dehydrogenase and malic enzyme assays, respectively. The reaction mixture for malate dehydrogenase activity contained 50 mM Tris-Cl, pH 7.4, 0.1 mM NADH, and 0.25 mM oxaloacetate. Activity was measured as the reduction in NADH which was monitored by decrease in absorbance at 340 nm. For malic enzyme, the assay medium contained 50 mM TES, pH 7.0, 0.4 mM $MnCl_2$, 0.1 mM $NADP^+$, and 0.4 mM L-malate. The increase in NADPH, which was observed as the increase in absorbance at 340 nm, was taken as malic enzyme activity.

In vitro Activation/Deactivation of PEPC

In vitro phosphorylation and dephosphorylation of alfalfa PEPC was achieved by incubating soluble protein extracts from non-acclimated or cold acclimated Anik seedlings with 5 mM ATP or alkaline phosphatase (0.05U/μg protein), respectively, at

30° C for 30 min. *In vitro* activation and deactivation was assessed by comparing PEPC activity after these treatments with controls incubated with water.

Electrophoresis, Immunoblotting and Immunoprecipitation

SDS-PAGE (10%) was performed using an established protocol (Laemmli, 1970). Resolved proteins were electroblotted onto a Polyscreen polyvinylidene fluoride (PVDF) transfer membrane (NEN/DuPont, Boston) in 192 mM glycine, 25 mM Tris, pH 8.3 (Towbin et al., 1979) using a Bio-Rad Trans-Blot cell. Protein concentrations and efficiency of transfer were verified by staining the membrane in 0.1% (w/v) Ponceau S in 1% acetic acid. The blot was incubated in blocking buffer (PBS containing 0.2% Tween) for 1 h to block non-specific binding sites on the membrane and then probed for 30 min at 37° C with anti-(alfalfa nodule PEPC) antiserum diluted in blocking buffer (1:4000 dilution). Unbound antibodies were removed with 4 changes of wash buffer (PBS containing 0.05% Tween) at 25° C. Immunoreactive polypeptides were recognized by incubation with horseradish peroxidase-linked goat anti-(rabbit immunoglobulin G) antibody (Transduction Laboratories, Lexington) diluted in blocking buffer (1:4000 dilution) for 30 min at 37° C, followed by 4 washes as above. Detection of immunoreactive bands was achieved using the Renaissance chemiluminescence system (NEN/DuPont, Boston) and/or colour development using diaminobenzidine as substrate. Immunoprecipitation experiments were carried out by incubating soluble protein extracts containing equal amounts of protein with mouse monoclonal antiphosphoserine antibody (Sigma, St. Louis) in a 1:200 dilution and placing on ice for

3h. The samples were then centrifuged at 15000 x g for 15 min and the precipitated proteins resuspended in SDS-sample buffer (Laemmli, 1970) and subjected to Western blotting using anti-PEPC as described above.

Protein determination

Soluble protein concentration was determined by the dye-binding method (Bradford, 1976) using the Bio-Rad reagents and BSA as a standard.

Non-autotrophic carbon fixation

Triplicate samples (1 g fresh weight) of non-acclimated or cold acclimated seedlings were incubated at 4° C in 100 mM Citrate buffer, pH 6.0, in a 1:4 (w/v) tissue:buffer ratio. Non-autotrophic carbon fixation was initiated with the addition of 3 μCi of $NaH^{14}CO_3$ (56 mCi/mmol) and the reaction allowed to proceed in the dark for 1h with shaking at 4° C. At the end of incorporation, the seedlings were washed 3 times with distilled water to remove unincorporated ^{14}C and the sample immediately frozen in liquid N_2 and stored at -80° C. Extraction was achieved by grinding the frozen samples in liquid N_2 in a chilled mortar and the powder obtained was transferred to a test-tube and weighed. The aqueous and organic phases were separated in chloroform:methanol:water and measurement of ^{14}C fixed in the acid-soluble fraction of the aqueous phase was determined as described by Ting and Dugger (1965b).

Analysis of PEPC transcript levels

Non-acclimated or cold acclimated Anik seedlings (1-2 g fresh weight) were harvested as for protein extraction. Isolation of total RNA and Northern blots were done as described by Monroy et al., 1993a. Blots were probed with a 405 bp product generated by RT-PCR (Monroy and Dhindsa, 1995). Primers used were the degenerate sense oligonucleotide 5'-TGCNTGGACACA(G/A)ACA(C/A)G(G/A)TT(T/C)-CA-3' and the complementary oligonucleotide 5'-ATCGCGGATCCGTTTCAATGTGTA-3', corresponding to nucleotides 2427 to 2450 and 2809 to 2832, respectively, of the alfalfa nodule PEPC cDNA sequence (Pathirana et al., 1992). Conditions for PCR were 94° C for 2.5 min and 80°C for 2 min for one cycle followed by thirty-five cycles of 94° C for 1 min, 60° C for 2 min and 72° C for 2 min.

RESULTS

PEPC activity and dark carbon fixation

Transfer of alfalfa seedlings (cv Anik) to 4° C for 4 d resulted in a 70% increase in the level of non-autotrophic carbon fixation (Fig. 1). Hence, enzymes involved in this process were tested for activity. There were no measurable changes in the activities of malate dehydrogenase or malic enzyme (data not shown), however, PEPC activity increased significantly with cold acclimation (Fig. 2). Although PEPC activity increased at least 100% with cold acclimation, this increase was more pronounced (250%) when assayed under suboptimal conditions, which were subsequently employed for all further PEPC assays.

PEPC protein and activity during cold acclimation

Cold acclimation affected both PEPC activity as well as the amount of protein that immunoreacted with an anti-PEPC antibody. PEPC activity increased during 4 d of cold acclimation to a maximum of 3.5-fold the activity of untreated plants (Fig. 3A). During the same period of cold acclimation, there was an increase in the amount of a 110 kD PEPC polypeptide and a decrease in the level of a 120 kD PEPC polypeptide (Fig. 3B). The minor immunoreactive bands could be PEPC degradation products or simply represent cross-reaction of the antiserum with unrelated polypeptides. This alteration in the levels of PEPC isoforms was not due to changes in the level of PEPC mRNA (data not shown). Due to the greatest difference in PEPC activity and protein

levels occurring after 4 d at 4° C, this length of cold treatment was used for all further experiments.

Comparison of PEPC in alfalfa cultivars differing in freezing tolerance

A comparison of PEPC activity and protein levels in the freezing-sensitive alfalfa cultivar Trek with the relatively freezing-tolerant cultivar Anik is shown in Figure 4. With cold acclimation, PEPC activity increased in both cultivars, although the difference was greater for Anik than for Trek (250% and 100%, respectively). Figure 3B reveals that the relative PEPC protein levels change in the same manner for both cultivars, in that the amount of the 120 kD band decreased and of the 110 kD band increased with cold acclimation. This suggests that factors other than the amount of the PEPC isoforms may be involved in causing the difference in PEPC activity that occurs with cold acclimation between Anik and Trek (Fig. 4A).

***In vitro* activation/de-activation of PEPC**

It has been found that seryl phosphorylation of PEPC is an important means of activating the enzyme in leaves of C_4, CAM and C_3 plants as well as in nodules of N-fixing legumes (Jiao and Chollet, 1991; Duff and Chollet, 1995; Zhang et al., 1995). *In vitro* experiments were carried out to investigate the role of reversible phosphorylation in regulating PEPC activity with cold acclimation in alfalfa seedlings. Figure 5 summarizes the results of experiments on *in vitro* phosphorylation or dephosphorylation of PEPC in soluble protein extracts obtained from non-acclimated or

cold acclimated Anik seedlings. Incubation with 5 mM ATP resulted in 29% and 34% activation of PEPC activity over the control levels for non-acclimated and cold acclimated extracts, respectively. In contrast, treatment with alkaline phosphatase resulted in a 67% and 72% reduction in PEPC activity for the non-acclimated and cold acclimated extracts, respectively. Thus, dephosphorylation of proteins in the cold acclimated extract by alkaline phosphatase reduced PEPC activity to the level of the non-acclimated control (Fig. 5). Similar *in vitro* results were obtained for Trek (data not shown). These findings suggest that the principle mechanism of regulating PEPC with cold acclimation is by altering its phosphorylation status.

***In vivo* phosphorylation status of PEPC and cold acclimation**

The *in vivo* phosphorylation status of PEPC in non-acclimated and cold acclimated seedlings of the alfalfa cultivars Anik and Trek was assessed by immunopreciptation. Soluble protein extracts were incubated with anti-phosphoserine antibody and an immunoblot analysis carried out on precipitated proteins using anti-PEPC antibodies. The only protein that was detectable after this procedure was the 110 kD polypeptide in the cold acclimated sample from Anik (Figure 6). This suggests that differential PEPC phosphorylation on serine residue(s) during cold acclimation accounts for the difference in cold-induced activity between Anik and Trek.

Figure 1: Effect of cold acclimation on dark carbon fixation in alfalfa seedlings (cv Anik). Samples of non-acclimated seedlings (NA) or seedlings cold acclimated for 4 d (CA) were incubated with $NaH^{14}CO_3$ for 1h in the dark and measurement of radiolabel incorporated into acid-stable products determined by liquid-scintillation counting as described in "Materials and Methods". The value of dark carbon fixation by non-acclimated seedlings, fixed as 100 %, was 34 cpm per μg protein per min. The results are given as the mean ± SE (n=6).

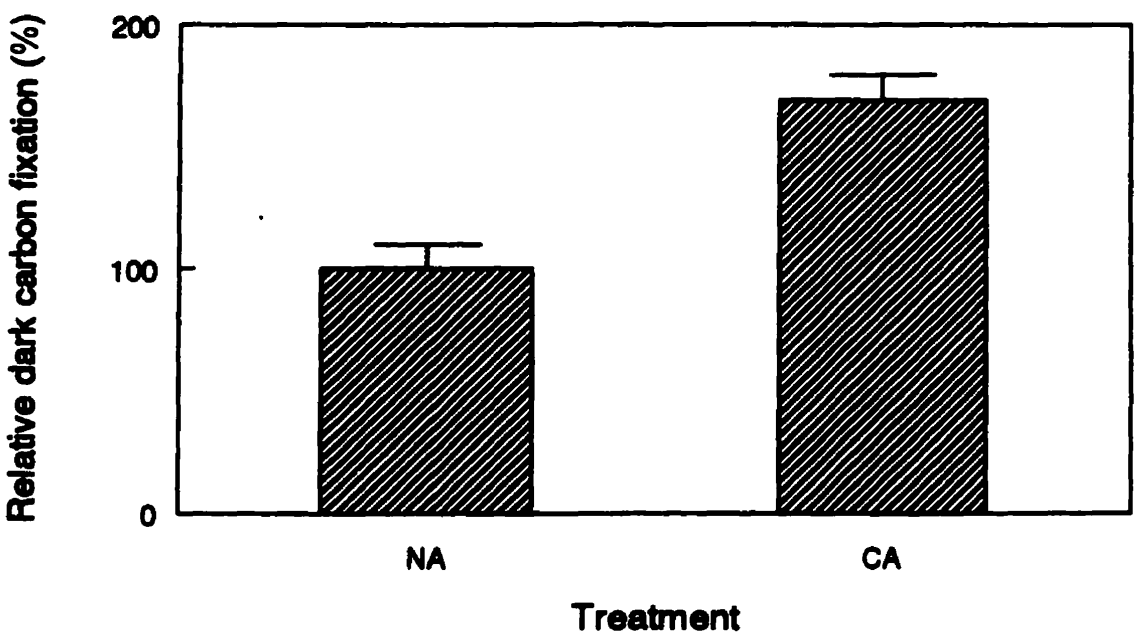

Relative dark carbon fixation (%)
200
100
0
NA
CA
Treatment

Figure 2: Effect of cold acclimation on PEPC activity in alfalfa seedlings (cv Anik). PEPC activity was assayed under optimal (pH 8, 2.5 mM PEP) or suboptimal (pH 7.5, 0.25 mM PEP) conditions in soluble protein extracts obtained from non-acclimated (NA) or cold acclimated (CA) seedlings. PEPC activity is expressed relative to the NA level for each condition which did not vary by more than 20% between experiments. The value for PEPC activity in non-acclimated seedlings was fixed as 1 and was 22 cpm per μg protein per min under suboptimal conditions and 35 cpm per μg protein per min under optimal conditions. The results are given as the mean ± SE (n=3).

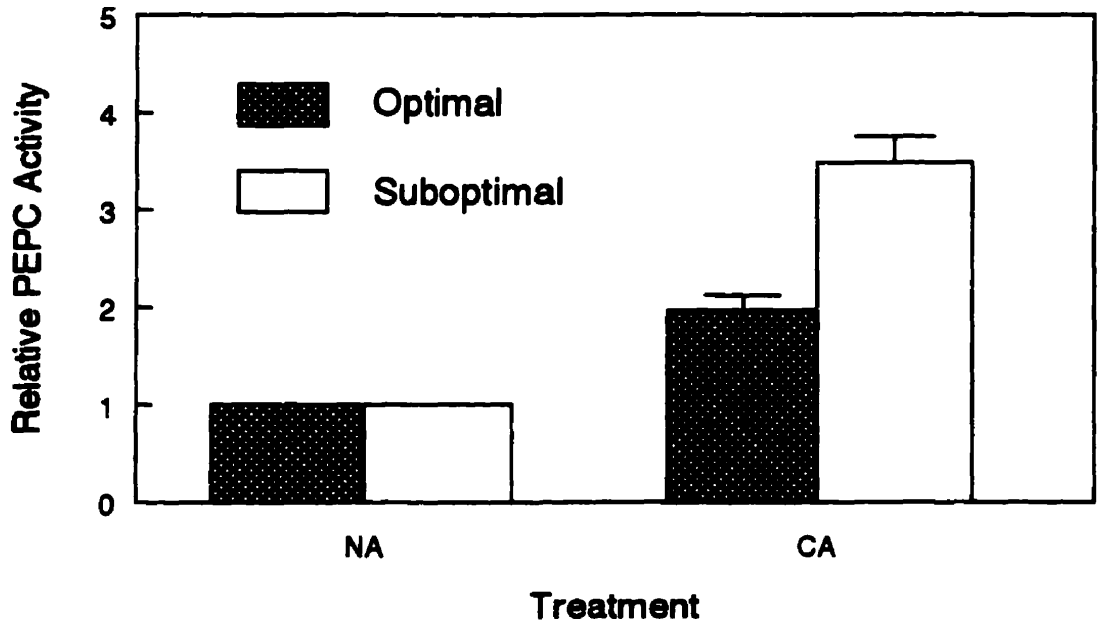
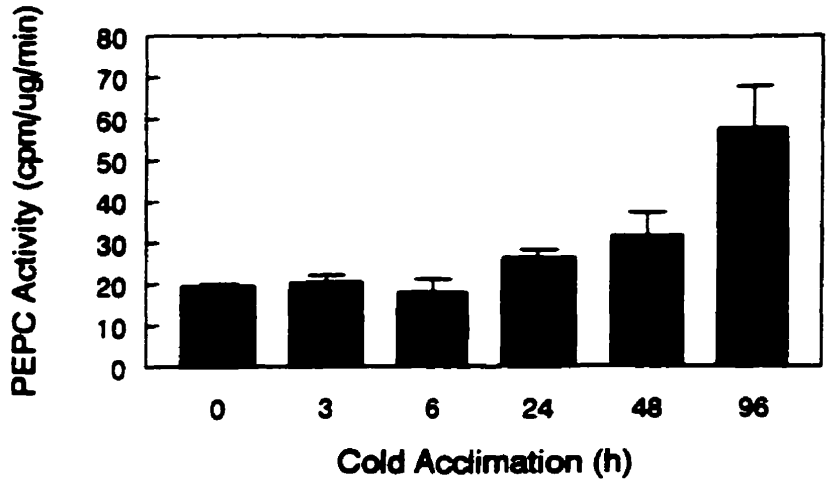

Relative PEPC Activity
5
4
3
2
1
0
Optimal
Suboptimal
NA
CA
Treatment

Figure 3: Time course of suboptimal activity (**A**) and protein levels (**B**) of soluble PEPC during cold acclimation. Alfalfa seedlings (cv Anik) were harvested after the indicated lengths of cold acclimation and soluble protein extracted. (**A**) PEPC activity was assayed at pH 7.5, 0.25 mM PEP and values represent the average of three separate experiments ± SE. (**B**) Soluble proteins (20 μg) were separated by SDS-PAGE, transferred to a polyvinylidene membrane, and probed with anti-alfalfa nodule PEPC antiserum. Immunoreactive bands were visualized as described in "Materials and Methods". The upper and lower arrows point to the 120 kD and 110 kD PEPC polypeptides, respectively.

A


PEPC Activity (cpm/ug/min)
80
70
60
50
40
30
20
10
0
0
3
6
24
48
96
Cold Acclimation (h)

B

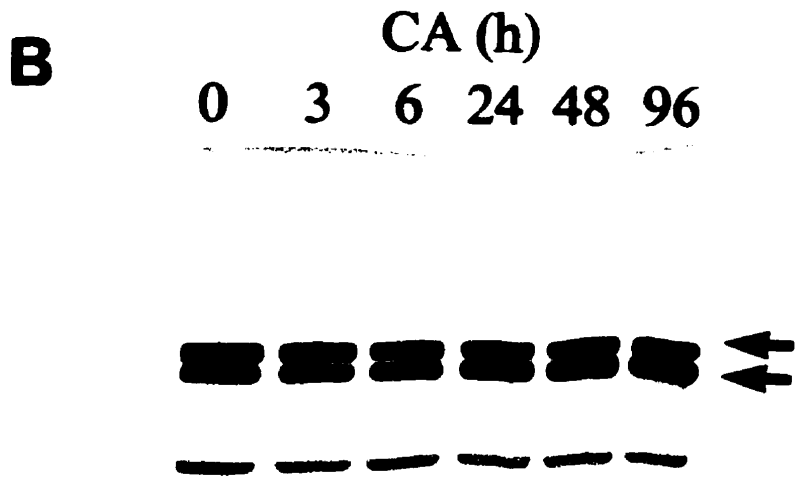
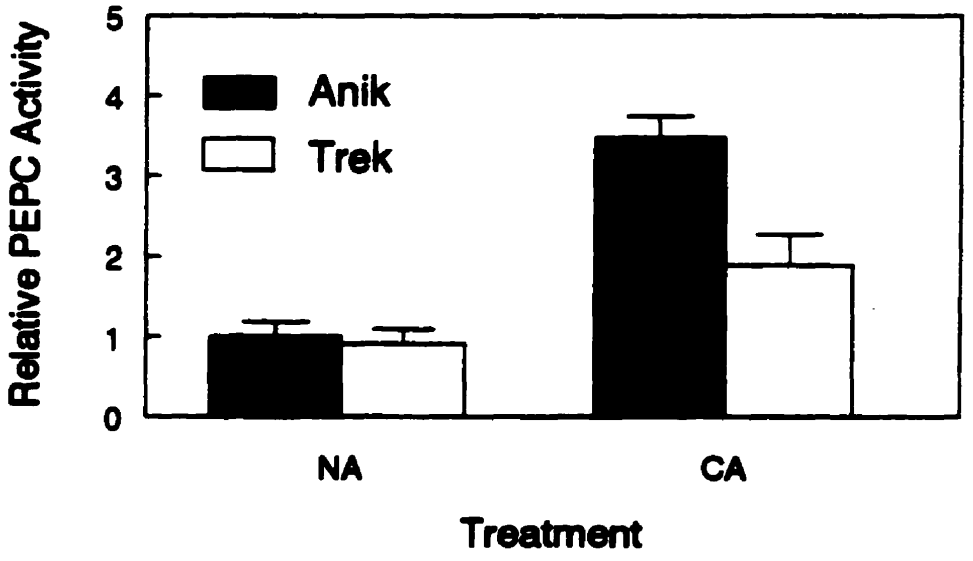

CA (h)
0 3 6 24 48 96

Figure 4: Suboptimal activity (**A**) and protein levels (**B**) of soluble PEPC during cold acclimation in two alfalfa cultivars differing in freezing tolerance. Soluble proteins were extracted from non-acclimated (NA) and cold acclimated (CA) seedlings of the freezing-tolerant cultivar Anik and the freezing-sensitive cultivar Trek. (**A**) The activity in non-acclimated seedlings was assigned the value of 1 which represented an activity of 114 cpm per μg protein per min in cultivar Anik, and 66 cpm per μg protein per min in cultivar Trek. PEPC activity was assayed under suboptimal conditions (pH 7.5, 0.25 mM PEP) and values represent the mean ± SE of at least five independent experiments. (**B**) Immunoblot analysis of soluble protein (25 μg) from NA and CA Anik and Trek seedlings was performed as described in Figure 2.

A


Relative PEPC Activity
5
4
3
2
1
0
Anik
Trek
NA
CA
Treatment

B

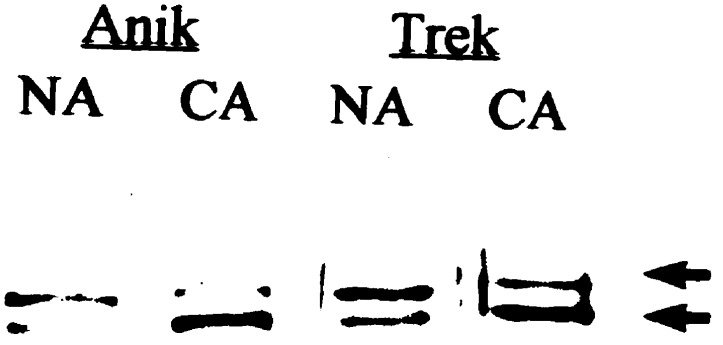

Anik
Trek
NA
CA
NA
CA

Figure 5: PEPC activity in non-acclimated (NA) or cold acclimated (CA) Anik protein extracts subjected to various treatments. PEPC was assayed under suboptimal conditions (pH 7.5, 0.25 mM PEP) following incubation with 5 mM ATP (+ATP), 0.05U/μg alkaline phosphatase (+AP), or H_2O (Control), at 30° C for 30 min. All values are expressed relative to control NA activity, which was 33 cpm per μg protein per min, and represent the means ± SE of three separate experiments.

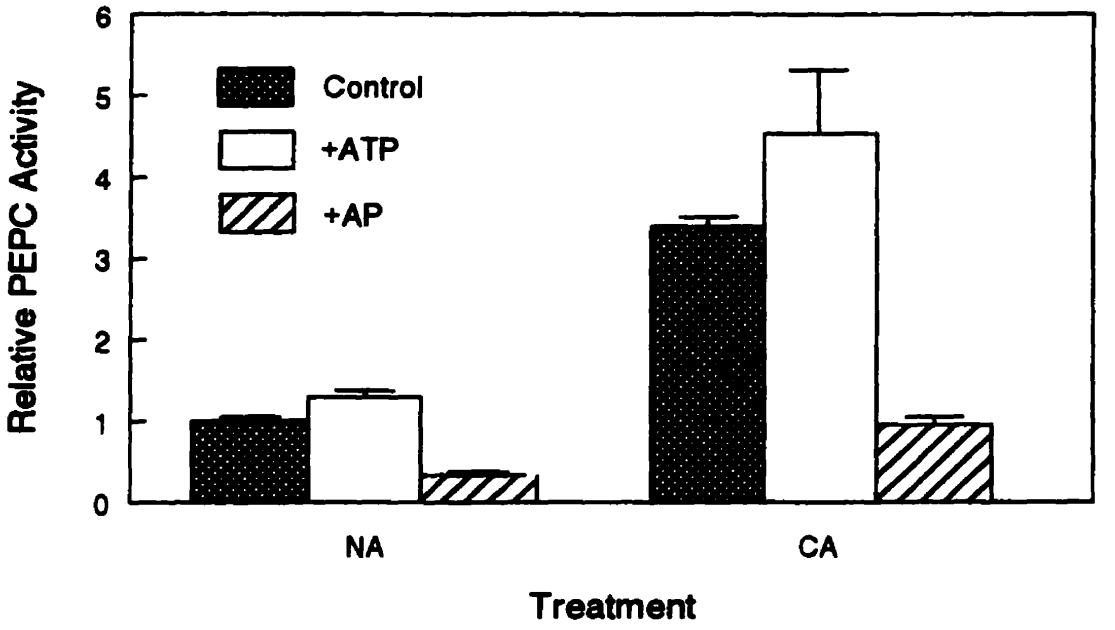
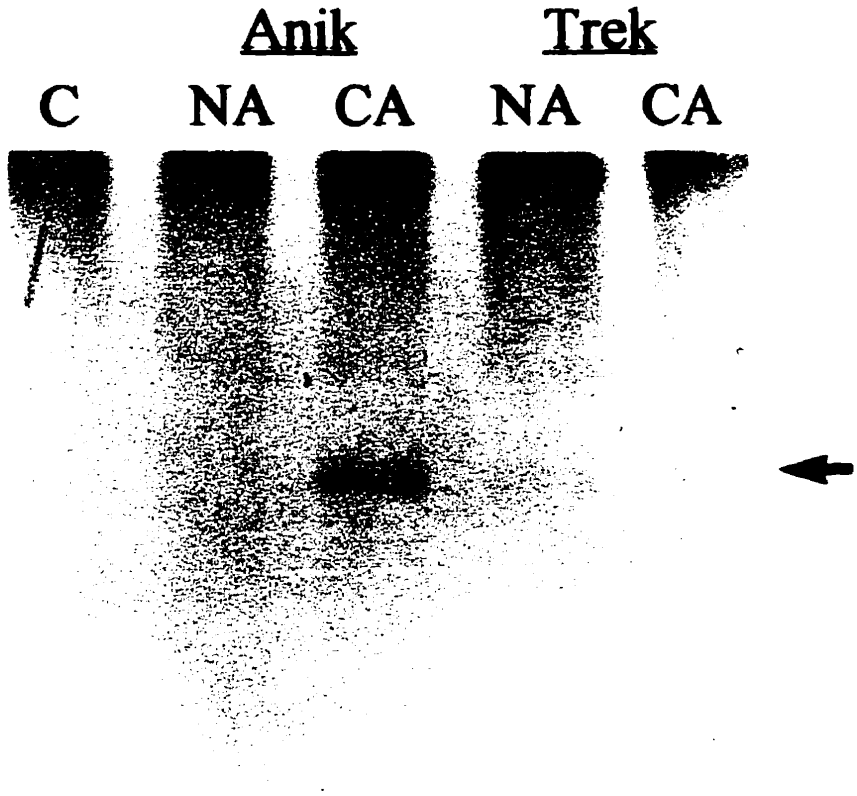

Relative PEPC Activity
6
5
4
3
2
1
0
Control
+ATP
+AP
NA
CA
Treatment

Figure 6: Immunoblot analysis of alfalfa PEPC preceded by immunoprecipitation with antiphosphoserine. Soluble protein (130 μg) extracted from non-acclimated (NA) or cold acclimated (CA) seedlings of alfalfa cultivars Anik and Trek was incubated with antiphosphoserine antibody (1:200 dilution) and immunoblot analysis carried out on precipitated proteins as described in Figure 2. The 110 kD immunoreactive polypeptide is indicated by an arrow. Lane C: Anik NA sample without antiphosphoserine.


Anik
Trek
C
NA
CA
NA
CA

DISCUSSION

The results of the present study demonstrate that there are changes in PEPC activity and protein levels that occur with cold acclimation, and that a freezing-tolerant (Anik) and a freezing-sensitive (Trek) cultivar of alfalfa differ with respect to these changes. The increase in PEPC activity that occurs with cold acclimation is paralleled with changes in the levels of PEPC isoforms in both Anik and Trek (Figs. 3A and 4). The roles of the different isoforms have not yet been elucidated, but it appears that the increase in the 110 kD monomer is responsible for at least some of the increased PEPC activity in Anik and perhaps all in Trek. The increase in level of the 110 kD subunit, which was greatest after 4d of cold acclimation (Fig. 3B), occurs despite no change in the level of PEPC mRNA (data not shown).

Throughout this investigation, we have chosen suboptimal pH and PEP for examination of PEPC activity, since it is well established that the phosphorylation status of PEPC is important when assaying under these conditions, but not when assaying under near-optimal conditions (Fig. 2) (Duff et al., 1995). We therefore exploited the enhanced activity of PEPC with cold acclimation when assayed under suboptimal, but physiologically relevant conditions (Schuller et al., 1990a), as an indicator of the apparent phosphorylation status of the enzyme in vivo.

The *in vitro* activation and deactivation experiments on Anik clearly establish that the phosphorylated PEPC is more active than the dephosphorylated form of the enzyme (Fig. 5). It is likely that phosphorylation of PEPC also decreases the enzyme's

sensitivity to inhibition by L-malate as has been found for the photosynthetic isoforms from C_4 and CAM plants (for a review, see Jiao and Chollet, 1991) as well as the nodule and C_3-leaf isoforms (Schuller and Werner, 1993; Duff and Chollet, 1995; Zhang et al., 1995). Finally, examination of the in vivo phosphorylation status of PEPC through immunoprecipitation with antiphosphoserine antibody followed by PEPC immunoblotting, revealed that the enzyme is seryl-phosphorylated in cold acclimated Anik seedlings but not in Trek or in non-acclimated plants (Fig. 6). This covalent modification most likely occurs near the protein's N-terminus at a target residue homologous to Ser^8 in sorghum or Ser^{15} in maize (Jiao et al., 1991a; Lepiniec et al., 1994), and explains the greater increase in PEPC activity that occurs with cold acclimation in Anik than in Trek. PEPC was partially activated by incubation with ATP suggesting that a protein kinase capable of phosphorylating PEPC is at least partially active in soluble protein extracts. It is not known if the PEPC kinase is a Ca^{2+}-independent serine/threonine kinase as has been found elsewhere (Echevaria et al.,1990; Li and Chollet, 1993; Duff and Chollet, 1995; Giglioli-Guivarc'h et al., 1996). However, these findings also demonstrate that the effects of cold acclimation on PEPC phosphorylation are complex and cannot be fully replicated in vitro (30 % in vitro activation compared to 250% in vivo increase in activity). Intracellular compartmentation is probably required to ensure site-specific protein-protein or allosteric interactions that regulate PEPC. It is also apparent that upstream events or parallel pathways are required to fully activate the enzyme including cytosolic alkalinization, Ca^{2+} mobilization, calcium-dependent protein kinases and protein synthesis (Pierre et

al., 1992; Giglioli-Guivarc'h et al., 1996). Previous findings in our laboratory have implicated roles for increased cytosolic calcium and protein kinases and phosphatases in low-temperature signal transduction during cold acclimation (Monroy et al., 1993a; Monroy and Dhindsa, 1995; Monroy et al., 1996).

The increased PEPC activity could account for the 70% increase in the level of non-autotrophic carbon fixation observed in cold acclimated seedlings of cultivar Anik (Fig. 1). This is in agreement with findings by other researchers who have attributed an increase in non-autotrophic carbon fixation to increased PEPC activity (Schuller et al., 1990b). Since malate levels remain unchanged during cold acclimation of alfalfa (LeBlanc and Dhindsa, unpublished data), malate is probably decarboxylated to pyruvate, thereby generating NADPH. Availability of NADPH during the early stages of cold acclimation may be useful to the cell in several ways. For example, NADPH is required for reductive biosynthesis of membrane components (Stumpf, 1980). Oxidative damage is common to several dehydrative environmental stresses such as drought (Dhindsa and Matowe, 1981) and cold (Clare et al., 1984; McKersie et al., 1993). A major consequence of the oxidative stress is the accumulation of oxidized glutathione which is known to inhibit protein synthesis in animals (Ernst et al., 1978) and plants (Dhindsa, 1987 and 1991). The regeneration of reduced glutathione and, therefore, the repair of oxidation damage depend on the availability of NADPH. It has been proposed (Vanlerberghe et al., 1989) that the reducing power generated by the non-autotrophic fixation of carbon may contribute to the maintenance of cellular redox balance.

In conclusion, to the best of our knowledge, the present study constitutes the first

report of regulatory phosphorylation of PEPC during cold acclimation. We have shown that an approximately 110 kD PEPC subunit is seryl-phosphorylated during cold acclimation in the freezing-tolerant alfalfa cultivar Anik, but not in the freezing-sensitive cultivar Trek. Moreover, this covalent modification is correlated with increased PEPC activity and indicates that during cold acclimation, PEPC is regulated in the same way as the enzyme from C_3, C_4 and CAM leaves, as well as nodules of N-fixing legumes. It should be of interest to characterize the signal transduction pathway, and particularly the protein kinases and phosphatases that are involved in the regulation of PEPC during cold acclimation.

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CONCLUSIONS

The results of the present study demonstrate that :

1) Cold acclimation of alfalfa results in an increased level of a 110 kD PEPC polypeptide and decrease in amount of a 120 kD PEPC monomer.

2) This shift in isoform levels occurs in both the freezing-tolerant cultivar Anik and the freezing-sensitive cultivar Trek and is associated with increased PEPC activity.

3) Cold acclimation results in a greater increase in PEPC activity in cultivar Anik than in cultivar Trek when assayed under conditions in which the phosphorylation status of the enzyme is important (250% and 100% increase, respectively).

4) PEPC is partially activated or deactivated as a result of in vitro phosphorylation or dephosphorylation, respectively.

5) The 110 kD PEPC subunit is seryl-phosphorylated with cold acclimation in the freezing-tolerant cultivar Anik, but not in the sensitive cultivar Trek. This may account for the greater cold-induced enhancement of PEPC activity in Anik than in Trek.

6) The increased PEPC activity could account for the increase in dark carbon fixation that occurs with cold acclimation of Anik seedlings.

Cold treatment results in an increased level of PEPC activity in both freezing-tolerant and -sensitive cultivars of alfalfa, although the enhancement is greater for the tolerant cultivar Anik. This is due to regulatory seryl-phosphorylation of a PEPC monomer that occurs with cold acclimation in Anik but not in Trek. This difference in regulation of enzyme activity between cultivars does not, in itself, indicate that this enzyme is responsible for imparting freezing tolerance to the plant. However, the finding that PEPC accounts for the increased level of dark carbon fixation during cold acclimation supports our hypothesis that the production of NADPH by this process may be useful towards cellular repair and development of freezing tolerance. It will be interesting to see if the enhancement of dark carbon fixation is the same for Trek as it is for Anik.

Future research might include examination of the signal transduction pathway activating PEPC to determine which, if any, signaling components play a role in regulation of the enzyme during cold acclimation. Of particular interest are the protein kinases and phosphatases that modify the enzyme and the determination of whether any activating/regulatory components are defective or absent in the freezing-sensitive cultivar. If so, then genetic manipulation may be possible to aid in the production of new strains of alfalfa which will be of great agronomic value to the Quebec and Canadian economies.

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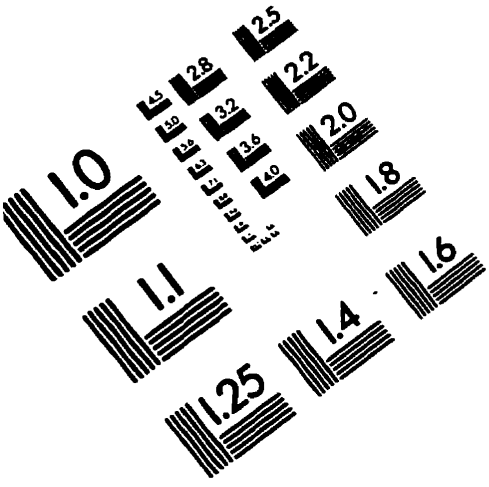

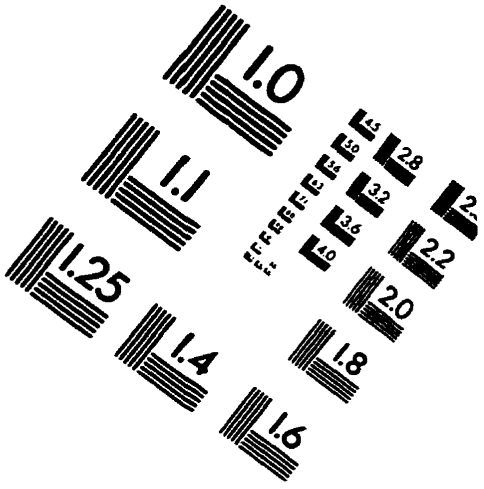

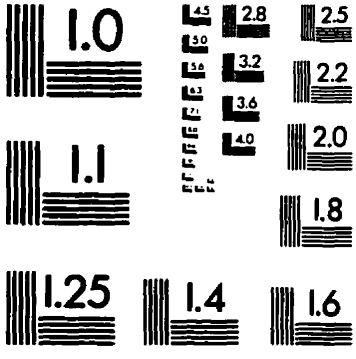

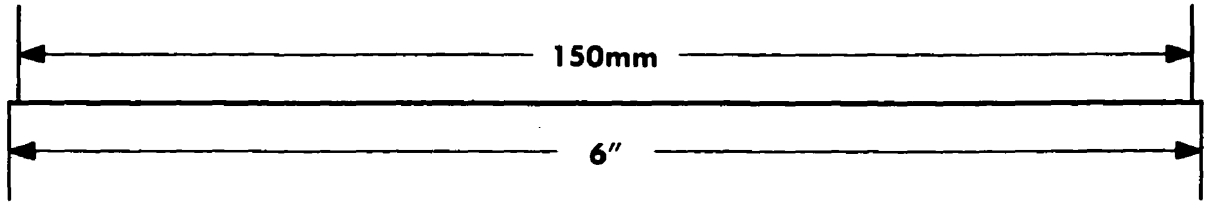

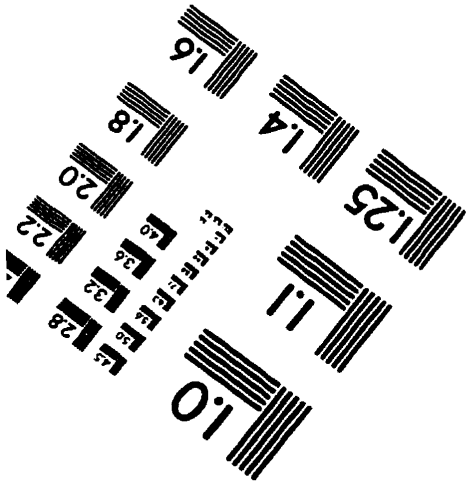

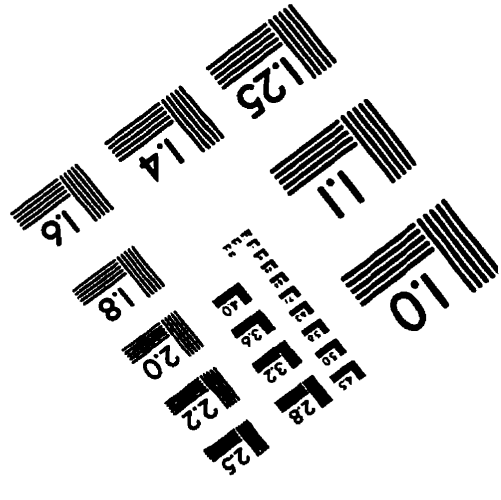

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