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Expression of *Chlamydomonas reinhardtii* periplasmic carbonic anhydrase in *Escherichia coli* and transgenic tobacco plants

by

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TABLE OF CONTENTS

ABSTRACT

Carbonic anhydrase (CA) catalyzes the reversible hydration of CO_2. The periplasmic CA gene *CAH1* of *Chlamydomonas reinhardtii* codes for a highly processed secreted glycoprotein. The primary translation product of the *CAH1* gene is targeted to the algae's ER, where it is proteolytically processed to yield two different subunits, glycosylated, assembled into an active heterotetramer, and secreted. Expression of *CAH1* in *Escherichia coli* and tobacco plants were effected in this study. For bacterial expression, two constructs, one with and another without, the algal target leader sequence were used. Similarly, after replacing the algal's target leader sequence with that from tobacco anionic peroxidase, expression of this gene in transgenic tobacco plants was also investigated.

No expression was detected in bacteria from the construct with the algal leader. The gene was however efficiently expressed in the cytoplasm of *E. coli* from the construct without the leader sequence. The expressed protein which accumulated to very high levels, was confirmed to be periplasmic CA 1 (peri-CA1) based on the expected size and by cross-reaction with polyclonal antibody. The overexpressed polypeptide was present in inclusion bodies and no CA activity was detected in cell homogenates. The band was excised from polyacrylamide gel, and after some processing, used to raise polyclonal antibodies in rabbits.

Of several transgenic plants screened by immunoblotting and found to express peri-CA1, a single plant, TL_1, with a high level of expression was chosen for further analysis. SDS-PAGE gels of the purified protein from this tobacco plant, showed that it migrated as a series of discrete bands (two large and one small) with slightly faster mobility than the comparable bands in the purified algal protein. The expressed protein in the transgenic plant was active, and staining with thymol and sulfuric acid confirmed that it was also glycosylated. Analysis of vacuum infiltrates from leaves expressing peri-CA1 showed that it was enriched in the intercellular fluid. The sensitivity of the enzyme to sulfonamide inhibitors was similar to that of the native algal enzyme. These results suggest that the post translational processing of *Chlamydomonas* peri-CA1 is largely conserved in a higher plant.

GENERAL INTRODUCTION

Rationale

Chlamydomonas reinhardtii is a unicellular, eukaryotic green alga. This micro-organism is capable of existing in different physiological states depending on the level of carbon dioxide in the growing "environment". At 5% level of CO_2, C_3 carbon fixation pathway with its attendant high CO_2 compensation point and photorespiration occurs. At air levels of CO_2, a CO_2 concentrating mechanism (CCM) is induced. Carbon fixation now occurs with the efficiency as prevails in C_4 in plants. The CO_2 compensation point is drastically reduced and photorespiration virtually eliminated. *CAH1* periplasmic carbonic anhydrase 1 (peri-CA1) is one of the genes induced in this mechanism.

The cellular metabolism of *Chlamydomonas*, for example as regards amino acid biosynthesis, fatty acid biosynthesis, ammonia assimilation and photosynthesis, is very similar to that as occurs in higher plants. If the genes of the CCM can be introduced into a C_3 plant, then it may be possible to similarly improve photosynthetic efficiency in plants, as happens in *Chlamydomonas*. Transforming tobacco plants with *CAH1* would be a first step in this direction. Additionally, we would be afforded the opportunity for assessing the expression of a *Chlamydomonas* gene in a higher plant, as the alga may serve as a source for obtaining "plant-like" genes. Further, because plant carbonic anhydrase and *Chlamydomonas* peri-CA1 evolved from different sources, it would be of biological interest to evaluate transcription, translation, precursor processing and enzyme activity of the algal gene in a higher plant. In other words, will the *Chlamydomonas* gene be similarly processed in the plant?

Goals of the study

This study was carried out to assess the expression of *C. reinhardtii* peri-CA1 in *Escherichia coli* and tobacco, cultivar Xanthi. Peri-CA1 is a secreted, glycosylated, heterotetrameric protein with intra- and intermolecular disulfide bonds, that is active in the periplasm of *C. reinhardtii*. We were interested in assessing expression in terms of

activity, assembly, and targeting of *Chlamydomonas* peri-CA1 to the apoplast of transgenic tobacco. Further, because of the extensive post-translational processing of peri-CA1 that occurs in *C. reinhardtii,* it was of interest to evaluate how much of this would be conserved by the higher plant. In order to be able to detect the expression and monitor the processing of peri-CA1 in the tobacco plant, an antiserum was needed. Since it was important that this antiserum not cross-react with oligosaccharide epitopes on other glycoproteins in transgenic tobacco plants, the antigen used for antibody production was the peri-CA1 expressed in *E. coli*, which completely lacks glycosylation. Additionally, expression in *E. coli*, allowed us to study in-vitro manipulation of the cytoplasmic expressed polypeptide to ascertain if it could be solubilized, denatured, then refolded to yield an active enzyme. Finally, expression in *E. coli* allowed us to ascertain if *C. reinhardtii CAH1* target leader sequence would function in the bacteria, and if an active product would result.

C. reinhardtii carbonic anhydrases

Carbonic anhydrase (CA) is a metallo-protein (containing zinc) that catalyzes the reversible hydration of CO_2 in water. CA has been found in plants and animals, the notable difference being that plant forms of the enzyme have a high molecular weight (MW), ranging from 140 kDa to 250 kDa, while in animals it is often around 30 kDa (Graham et al., 1984). The enzyme is widespread in nature: it has been found in bacteria, cyanobacteria, green, red and brown algae, ferns and higher plants (Reed and Graham, 1981). Most plant CAs have one Zn atom per subunit (Graham et al., 1984). *C. reinhardtii* is a unicellular, eukaryotic, green alga with CA activity in the cytosol, chloroplast (Moroney et al., 1987; Husic et al., 1989; Sultemeyer et al., 1990), and periplasm (Kimpel et al., 1983; Coleman et al., 1984; Yang et al., 1985). There are two periplasmic CA genes in *C. reinhardtii*, *CAH1* and *CAH2*. Induction of all forms of CA occurs when *C. reinhardtii* cells are transferred from growth at high CO_2 to air (Badger and Price, 1994) except for *CAH2*, transcripts of which decrease with limiting inorganic carbon (C_i) (Fujiwara et al., 1990; Fukuzawa et al., 1990). At air levels of CO_2, an active CCM is induced, whereby CO_2 can be concentrated internally to a level 40-fold higher than exists in the media (Badger et al., 1980). At these limiting C_i levels, *CAH1* mRNA increases (Fujiwara et al., 1990; Fukuzawa et al., 1990) and peri-CA1 is expressed at very

high levels, accounting for 80 to 95% of CA activity (Kimpel et al., 1983; Coleman et al., 1984; Yang et al., 1985).

Peri-CA1 is synthesized as a 41.6 kDa precursor polypeptide which is processed post-translationally (Toguri et al., 1986; Fukuzawa et al., 1990; Kamo et al., 1990; Coleman, 1991). The mature holoenzyme is a heterotetrameric glycoprotein, consisting of two large and two small subunits (see Figure 1), that is secreted to the periplasmic space of the alga (Kamo et al., 1990). There are a number of inter- and intramolecular disulfide bonds in the holoenzyme (Ishida et al., 1993) (Figure 1).

Plant carbonic anhydrases

In C_3 plants, CA is an abundant protein, predominantly located in the stroma of chloroplasts, (Jacobson et al., 1975; Tsuzuki et al., 1985), even though cytosolic forms have been suggested (Reed and Graham, 1981; Graham et al., 1984). CA of C_4 leaves occur almost exclusively in the cytosol of the mesophyll cells (Burnell and Hatch, 1988). There are two distinct types of the enzyme among higher plants, one of high MW found in dicots and the other of low MW in monocots (Atkins et al., 1972a; Atkins et al., 1972b). CA has been characterized in two monocots, *Tradescantia* and barley. The MW was 42.0 kDa in the former and 45.0 kDa in the latter (Reed and Graham, 1981). Generally, the native enzyme in dicotyledon species is a hexamer of identical subunits. Each subunit has a MW 26.5 to 30.0 kDa and contains one zinc atom associated with the active site (Pocker and Ng, 1973). Plant CAs are much less sensitive to sulfonamides (acetazolamide, sulfanilamide and ethoxyzolamide) than animal CAs (Reed and Graham, 1981). It is thought that these inhibitors function by associating with the zinc atom in the CA enzyme, thus it is suggested that the access "tunnel" to the active site is smaller in plants versus animals (Reed and Graham, 1981).

The precise role of CA in plants is not clear, though it is speculated to be involved in photosynthesis. In C_3 plants, a possible role is that its presence in the stroma aids transport of C_i across the chloroplast by facilitated diffusion (Raven and Glidewell, 1978; Cowan, 1986; Badger and Price, 1994). It has also been suggested that CA may play a role in CO_2 fixation with Rubisco (Bird et al., 1980; Reed and Graham, 1981; Majeau and Coleman, 1994). In C_4 plants carbonic anhydrase converts CO_2 entering the

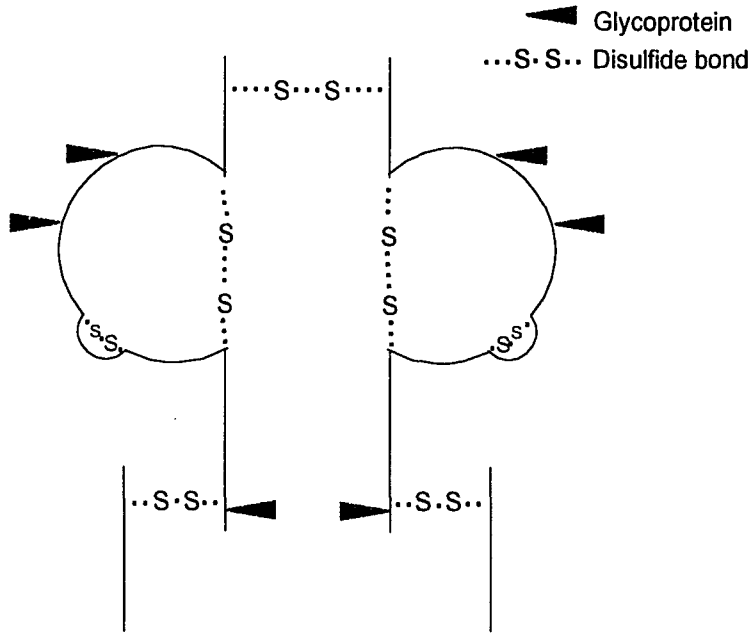

Figure 1. Line drawing of *C. reinhardtii* periplasmic CA showing the two large subunits, two small subunits, intermolecular disulfide bond between the large subunits, two intramolecular disulfide bonds within the large subunits, intermolecular disulfide between the large and small subunits, and three glycoproteins on each large unit.

mesophyll cells to HCO_3^-, the substrate for phosphoenolpyruvate carboxylase, in the first step in C_4 photosynthesis (Hatch and Burnell, 1990).

Comparison of *Chlamydomonas* versus plant CA and their evolution

The two periplasmic CA genes in *C. reinhardtii*, *CAH1* and *CAH2*, show 92% sequence homology (Fujiwara et al., 1990; Fukuzawa et al., 1990). The genes are structurally related, occurring in a tandem cluster on the genome (Fujiwara et al., 1990). They are thought to have evolved as a result of duplication of the CA gene in the *Chlamydomonas* genome (Fujiwara et al., 1990). They show greater homology with mammalian CAs (especially around the active site) than with plant chloroplast CAs (Fujiwara et al., 1990; Fukuzawa et al., 1990).

Plant chloroplast CA cDNA sequences from spinach (Burnell et al., 1990), pea (Roeske and Ogren, 1990; Majeau and Coleman, 1991) and tobacco (Majeau and Coleman, 1992) have been characterized. Fukuzawa et al. (1992) reported that chloroplast CAs from C_3 plants show a high degree of sequence similarity (approximately 76%) to each other, and that cytosolic CA from maize had a 60% sequence similarity to chloroplast CAs. Comparing the amino acid sequence of the mature subunits of periplasmic CA with human CA isozymes, Fukuzawa et al., (1990) found 20.4% sequence homology with CAI, 21.8% with CAII and 20.7% with CAIII.

Fukuzawa et al., (1992) isolated a putative CA gene, *icfA*, from cyanobacteria *Synechococcus* PCC7942. The deduced amino acid product of this gene shows significant sequence similarity with chloroplast CAs from pea (22%), spinach (22%) and *Escherichia coli* (31%). The cyanobacteria *icfA* gene product and plant chloroplast CAs however, have no significant sequence similarity with mammalian CA isozymes (Hewett-Emmett and Tashian, 1991) or *Chlamydomonas* periplasmic CAs (Fujiwara et al., 1990; Fukuzawa et al., 1990).

It would thus appear that *Chlamydomonas* periplasmic CAs and human CA isozymes share a common ancestry, but that *Chlamydomonas* evolved differently thereby acquiring the post-translational processing that split the protein into different subunits (Fukuzawa et al., 1990). Plant chloroplast CAs may have evolved from a common ancestor, which is however of another distinct source from *Chlamydomonas* periplasmic CA (Fukuzawa et al., 1992).

Protein secretion and post-translational processing

Proteins that will be localized within endomembrane compartments must possess signals targeting their entry into the endoplasmic reticulum (ER). Such targeting is generally accomplished via transit peptides at the amino terminus of the polypeptide. The polypeptide is translocated co-translationally across the ER membrane and into the lumen. Transport of proteins into the eukaryotic secretory pathway follows the vectoral route: ER → Golgi → vacuole/lysosomes/plasma membrane (Walter and Lingappa, 1986). During or after the process of translocation across the membrane, the polypeptide may acquire secondary, tertiary and/or quarternary structures. It may be further modified by the addition of glycans, cleavage by proteolytic enzymes and the formation of disulfide bonds within or between polypeptides (reviewed by Vitale et al., 1993, and references therein). Sorting of proteins to the vacuole or retention in the ER are thought to be controlled by "signal sequences", though these are not always clearly defined (reviewed by Bennett and Osteryoung, 1991). In mammals and other higher eukaryotes, many active secreted proteins are proteolytically cleaved from inactive precursor polypeptides by the sequential activities of at least two specific proteases: a dibasic endoprotease, and an exopeptidase with specificity for COOH-terminal basic amino acid residues (Hutton, 1990). Secretion from the cell is due to fusion of Golgi-derived and possibly ER-derived vesicles with the plasma membrane (Akazawa and Hara-Nishimura 1985).

Methods of investigation

To facilitate this study, polyclonal antibodies were raised using two sets of antigens. Affinity purified peri-CA1 from *C. reinhardtii* was one antigenic source, while the peri-CA1 expressed in *E. coli*, was the other. A number of plasmids were constructed to facilitate expression in *E. coli* bacteria and tobacco cultivar Xanthi. The open reading frame of *CAH1*, with and without the native target leader sequence, was cloned into bacterial expression vectors. For plant expression, the tobacco anionic peroxidase leader sequence (Lagrimini et al., 1987) was included in constructs in order to facilitate targeting

to the ER. Expression was detected via immunoblotting. The expressed protein was characterized molecularly and biochemically.

Principal results

CAH1 was efficiently expressed in *E. coli*, accounting for a significant amount of the total bacterial cell protein 4.5 hours after induction. The polypeptide was localized to the cytoplasm in inclusion bodies. None was detected in the periplasmic space. That it was peri-CA1 was confirmed via it being the anticipated size (approximately 39 kDa) and cross reaction with polyclonal antibody raised from the algal protein. The over-expressed band was identified on a Coomassie brilliant blue stained SDS-PAGE gel. The band was excised from the gel, and after some processing, used to raise polyclonal antibodies. No CA activity was detected in the bacterial cell homogenates, after induction. Additionally, the overexpressed polypeptide was insoluble. Similarly, after replacing the target leader sequence with that from tobacco anionic peroxidase, *CAH1* was highly expressed in tobacco plants. Anti-periplasmic CA cross-reacted with polypeptides in total plant protein extracts, confirming expression of the algal gene in the transgenic plants. The purified protein from transgenic plants migrated as a series of discrete bands (two large and small "doublet") on SDS-PAGE with slightly faster mobility than the comparable bands in the purified algal periplasmic CA. The expressed protein in the plant was active, glycosylated and secreted. The activity of the enzyme was similarly inhibited as it is in *Chlamydomonas*, by acetzolamide and ethozyolamide.

Dissertation organization

This dissertation details the expression of *C. reinhardtii* peri-CA1 in *E. coli* and tobacco (*Nicotiana tabacum* cv. Xanthi) plants. The bacterial expression system was used to study expression in *E. coli* and to produce a non-glycosylated form of the enzyme, which was thought "most prudent" for antibody production (Chapter 2). This polyclonal antibody was then used extensively in analyzing expression of the algal gene in transgenic tobacco plants (Chapter 3).

The dissertation consists of an introduction, two chapters detailing the experiments done and results obtained, and a brief summary. References cited in the introduction are listed at the end of this chapter. It is intended that the work reported in Chapter 3 be submitted for publication. Cyril S. Roberts was the principal researcher responsible for conducting this work, while Dr. Martin H. Spalding served as the major professor.

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EXPRESSION OF *Chlamydomonas reinhardtii* PERIPLASMIC CARBONIC ANHYDRASE IN *Escherichia coli*

Overview

Carbonic anhydrase (CA) catalyzes the reversible hydration of CO_2. There are two periplasmic CA genes in *Chlamydomonas reinhardtii, CAH1* and *CAH2. CAH1* codes for a protein that is a secreted, glycosylated, heterotetramer. It was of interest to express *CAH1* in *Escherichia coli*, to ascertain if an active product would result and whether it would be secreted to the bacteria's periplasmic space. It was also desirable to generate antigen for preparing polyclonal antibody to this protein, for use in identifying the expressed product in transgenic plants. Antibody so generated would not cross-react with oligosaccharide epitopes on other glycosylated proteins present in plants. Such antiserum would also be effective to follow processing of the gene in transgenic tobacco. It was thought that cytoplasmic expression of the gene would best accomplish this objective. Additionally, attempts were made to manipulate the cytoplasmic product *in-vitro* to achieve solubility and possibly activity.

The gene was efficiently expressed using T7 RNA polymerase/T7 promoter expression systems in the cytoplasm, however, the polypeptide was not detected in the periplasmic space. An over-expressed band visualized on an SDS-PAGE gel stained with Coomassie brilliant blue. It was confirmed to be periplasmic CA 1 (peri-CA1) based on the expected size (approximately 39 kDa) and by cross-reaction with polyclonal antibody raised from the algal protein. The expressed protein accumulated to very high levels, accounting for a significant proportion of the total bacterial cell protein 270 minutes after induction. No CA activity was detected in cell homogenates, after induction. Additionally, the overexpressed polypeptide was insoluble and efforts to solubilize it proved unsuccessful. The band was thus excised from the polyacrylamide gel, and after some processing, used to raise polyclonal antibodies in New Zealand white rabbits.

Introduction

Carbonic anhydrase (CA) is widespread in nature. It is a metallo-enzyme that contains zinc at the active site. The enzyme catalyses the reversible hydration-dehydration reactions:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

Two different periplasmic CA genes, *CAH1* and *CAH2*, have been reported in *Chlamydomonas* (Fujiwara et al., 1990). *CAH1* is highly expressed when the cell is exposed to light and limiting external inorganic C (C_i). *CAH2* is apparently down regulated by limiting external C_i and is not highly expressed under any condition; it encodes another periplasmic CA isozyme made under high CO_2 levels (Fujiwara et al. 1990). For purposes of the work described in this chapter, *CAH1* is the gene of interest as it is induced and its product (peri-CA1) accumulates during induction of the CO_2 concentrating mechanism (CCM) of *Chlamydomonas*.

The CCM is a means whereby *Chlamydomonas* can concentrate CO_2 internally 40-fold higher than exists externally (Badger et al., 1980). The CCM allows this C_3 alga to carry out photosynthesis with C_4-like efficiency. *CAH1* periplasmic CA is an inducible component of the CCM in *Chlamydomonas reinhardtii*. The level of its mRNA increases within 1 hour of shifting from high (5%) levels of CO_2 to ambient (0.04%) levels (Fujiwara et al. 1990, Fukuzawa et al. 1990). The enzyme accumulates to very high levels in the periplasmic space, accounting for 80 to 95% of the CA activity (Kimpel et al. 1983, Coleman et al. 1984, Yang et al. 1985). It probably contributes to the higher affinity for C_i at air levels of CO_2 during operation of the CCM, by converting HCO_3^- to CO_2, which can be taken up through the plasma membrane and then used in photosynthesis (Badger and Price, 1994).

CAH1 encodes a 377 amino acid polypeptide (41,626 daltons) (Fukuzawa et al. 1990). It consists of a 20 amino acid hydrophobic signal peptide at the amino terminus, a large subunit of 35,603 daltons and a small subunit of 4,144 daltons. These subunits are cotranslated as a precursor polypeptide (Fujiwara et al. 1990, Fukuzawa et al. 1990). There are three consensus sequences (Asn-Xaa-Thr/Ser) for *N*-glycosylation sites in the large subunit, which are all glycosylated (Fukuzawa et al. 1990, Ishida et al. 1993). It is estimated that the carbohydrate content of the mature protein is 13%, or approximately

4.8 kDa (Toguri et al., 1986). Gradient SDS-PAGE reveals a doublet of bands (35 to 37 kDa) for the large subunits, however, both bands have the same *N*-terminal sequence, at least up to the 10th amino acid (Kamo et al. 1990). Chemical deglycosylation of the mature enzyme results in only one 32 kDa polypeptide (Toguri et al. 1986), suggesting that the two large subunit bands result from differential glycosylation of the large subunit. The holoenzyme is a heterotetramer (Kamo et al. 1990) and has a number of intermolecular (three) and intramolecular (two) disulfide bonds (Ishida et al. 1993).

A major goal of this work was to determine whether a CA polypeptide would be secreted to the periplasmic space of the bacteria using the native *CAH1* leader sequence and remain active. Another area of interest was the generation of non-glycosylated *Chlamydomonas* peri-CA1 in abundant quantities for use in polyclonal antibody production. The antiserum produced could be used to detect *C. reinhardtii* peri- CA1 expression and processing in transgenic tobacco plants. It should be able to recognize the prepolypeptide, large subunits, small subunits, leader and "spacer" region of periplasmic CA during post-translational processing. Additionally, such antiserum would have very little likelihood of cross-reacting with oligosaccharide epitopes on other glycoproteins in transgenic tobacco plants. Finally, the *in-vitro* manipulation of the cytoplasmically expressed polypeptide, to study if it can be solubilized, denatured, then refolded to yield active product was also of interest.

Materials and methods

Plasmid constructions

A full length cDNA clone of *CAH1*, designated pCA, was obtained by taking advantage of the published sequence by Fukuzawa et al (1990) to extend a partial cDNA clone (Spalding et al., 1991) to full length by using PCR (Winder, 1991). To facilitate use of the *Nco I* site at the translation start site (+1) in vector construction, the *Nco I* site at position +123 was eliminated by changing His^{42} codon from CAT to CAC (Winder, 1991). Using the site-directed mutagenesis method of Kunkel et al. (1985), a *Hpa I* site was added at +1138 after the stop codon by changing the "C" at +1136 to a "G", and "CC" at +1139, +1140 to "AA". This construct was designated pCA-Hpa. Next, an *Nco I* site was introduced at +53, between the leader sequence and the large subunit. This

resulted in Ala^{18} GCG being changed to GCC, and Gln^{19} codon CAG being replaced with Met codon ATG. This second construct was designated pCA-Hpa (5'Nco). A 26-mer (Oligo-1) spanning +1125 to +1150 was used to effect mutagenesis in the former, while a 27-mer (Oligo-2) spanning +43 to +69 was used in the latter (see Figures 1 and 2).

pCA-Hpa (leader) and pCA-Hpa (5'Nco) (no leader) were first cut with *Hpa I*, then *BamH I* linkers ligated to the blunt ends (Sambrook et al. 1989), followed by cutting with *Nco I* and *BamH I*. The resulting open reading frame inserts with *Nco I* 5' and *BamH I* 3' ends were then ligated with the *Nco I/BamH I* ends of the *Escherichia coli* expression vector pET11d (Novagen), which carries the bacteriophage T7 ϕ10 promoter and ϕ Terminator. The completed plasmids were designated pET11d-CA-CL (leader present) and pET11d-CA (no leader) (see Figure 3). Plasmids pET11d-CA-CL and pET11d-CA were used to transform bacterial host HMS174 (DE3), an *Escherichia coli* K12 strain (F^- *recA* r_{k12}^- m_{k12}^+ Rif^r) lysogenized with a lambda derivative, DE3. DE3 carries the immunity region of phage 21, the *lacI* gene, the *lac*UV5 promoter, the start of the *lacZ* gene and the gene for T7 polymerase inserted into the *int* region of the phage (Studier and Moffat, 1986). The lacUV5 promoter directs transcription of T7 RNA polymerase upon the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Studier et al., 1990).

Expression

Expression was carried out as described by Sano and Cantor (1990) with a few modifications. The HMS174(DE3) bacterial host carrying the target plasmid was grown in a 50 ml culture at 37° C with shaking in 2X YT medium (Sambrook et al. 1989) supplemented with 50 μg/ml ampicillin. When the A_{600} reached 0.6, a 1 ml sample was taken before adding IPTG to a final concentration 1 mM to induce the T7 RNA polymerase gene under the lacUV5 promoter. The culture was allowed to grow a further five hours, with 1 ml samples being taken for analysis of heterologous protein at 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes after IPTG induction. Cells from 1 ml of the above culture were microcentrifuged for 60 seconds at 4° C. The supernatant was

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                                                    GAGTCATTACCTGCAACCCACTTGAACACC   -1
                                                                              CAT
+1  ATGGCGCGTACTGGCGCTCTACTCCTGGTCGCGCTGGCGCTTGCGGGCTGCGCGCAGGCT                  60

61  TGCATCTACAAGTTCGGCACGTCGCCGGACTCCAAGGCCACCGTTTCGGGTGATCACTGG                  120
        C
121 GACCATGGCCTCAACGGCGAGAACTGGGAGGGCAAGGACGGCGCAGGCAACGCCTGGGTT                  180

181 TGCAAGACTGGCCGCAAGCAGTCGCCCATCAACGTGCCCCAGTACCAGGTCCTGGACGGG                  240

241 AAGGGTTCCAAGATTGCCAACGGCCTGCAGACCCAGTGGTCGTACCCTGACCTGATGTCC                  300

301 AACGGCACCTCGGTCCAAGTCATCAACAACGGCCACACCATCCAGGTGCAGTGGACTTAC                  360

361 AACTACGCCGGCCATGCCACCATCGCCATCCCTGCCATGCACAACCAGACCAACCGCATC                  420

421 GTGGACGTGCTGGAGATGCGCCCCAACGACGCCGCCGACCGCGTGACTGCCGTGCCCACC                  480

481 CAGTTCCACTTCCACTCCACCTCGGAGCACCTGCTGGCGGGCAAGATCTATCCCCTTGAG                  540

541 TTGCACATTGTGCACCAGGTGACTGAGAAGCTGGAGGCCTGCAAGGGCGGCTGCTTCAGC                  600

601 GTCACCGGCATCCTGTTCCAGCTCGACAACGGCCCCGATAACGAGCTGCTTGAGCCCATC                  660

661 TTTGCGAACATGCCCTCGCGCGAGGGCACCTTCAGCAACCTGCCGGCGGGCACCACCATC                  720

721 AAGCTGGGTGAGCTGCTGCCCAGCGACCGCGACTACGTAACGTACGAGGGCAGCCTCACC                  780

781 ACCCCGCCCTGCAGCGAGGGCCTGCTGTGGCACGTCATGACCCAGCCGCAGCGCATCAGC                  840

841 TTCGGCCAGTGGAACCGCTACCGCCTGGCTGTGGGCCTGAAGGAGTGCAACTCCACGGAG                  900

901 ACCGCCGCGGACGCCGGCCACCACCACCACCACCGCCGCCTGCTGCACAACCACGCGCAC                  960

961 CTGGAGGAGGTGCCTGCCGCCACCTCCGAGCCCAAGCACTACTTCCGCCGCGTGATGCTG                 1020

1021 GCCGAGTCCGCGAACCCCGATGCCTACACCTGCAAGGCCGTTGCCTTTGGCCAGAACTTC                 1080
                                                                  G  AA
1081 CGCAACCCCCAGTACGCCAACGGCCGCACCATCAAGCTGGCCCGCTATCACTAAACTTCC               1140

1141 CAGTAGTTAGTCACGCTACCACCGTCGGCACGGCCAGCAGGCATTCCATTTTCCAGGCTT                 1200

1201 TGCTTCACGGTTTGGTGTGTCATTCGATGGTGTTCTTGACGACCCGCGCTTGGCGGGCCT                 1260
1261 TTCCAATTTTTTCCATAGTACACCGAAATAGTTCTGCGGTGCAGCACGCATACACACAGT                 1320
1321 ACCGGACGGGCGGCGGGACCTCCTGTTTTCTCCTGACTAGTAAAGAAGTAAGGAAGGTAT                 1380
1381 GGAGTTGGTTCCACGATGGGGCAGTCTGAGAGCGGAATAAAGTCAGTGGGCCGGACGTTG                 1440
1441 TGGCGATGGATGGTAGTGAGGCAAGTAATACGTACGTAGAGGGCGTACGCGGGTAATAAC                 1500
1501 GGGAACTTCGACAGCAATCGAGAGTGTCTGCACGCGAGACATTTGCGTACAGGGGAGGCA                 1560
1561 CCGGTTCTCCTCGATGAGTGATCCGTACTTATGCAAGTTATATAAGGCTGGTGTGGGGCG                 1620
1621 CTTCAGCACGGTATGGTTGCCAGCATGCACGGTCCGGCCTCTGTCTGGCTGGCTGGGTTG                 1680
1681 TTGGCGGGCTGGCCTCATGCGCGCCGTCGCACATGCCGATCAATGCAGTTGCTCTCCAGT                 1740
1741 AGCTGCAAGGCCTGGCTGGGCAATCCCATAGCCATGTCGAATGTGAAGCATTGTTTTCTT                 1800
1801 GGAGATGGAGGACAGGAGACGCTGACCGGATGTTTTAAGACGTGCAGGATGTGGGGAGCG                 1860
1861 AGGTAGCTACAACGGTGCAGTTGAGGCAGAGACGTGTACGACATGTAAGATGCCCATGGA                 1920
1921 CAAAAAAAAAAAAAAAAAAAA                                                      1942
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Figure 1. cDNA nucleotide sequence of *Chlamydomonas reinhardtii* periplasmic CA showing bases changed (**bold-face**), new bases substituted (*italic*) via site directed mutagenesis, along with Oligo I and Oligo II used (<u>underlined</u>).

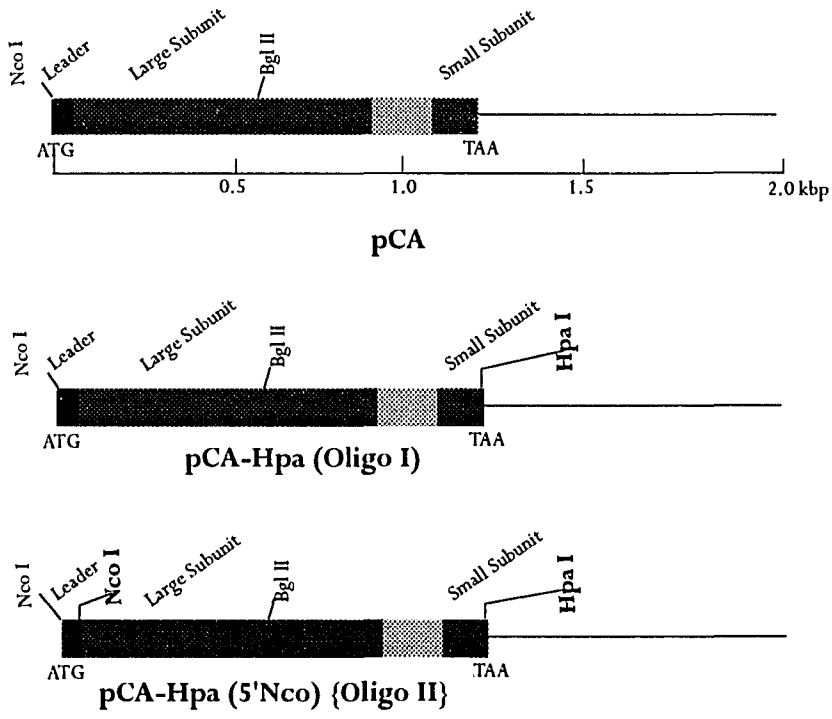

Figure 2. Site directed mutagenesis of pCA with Oligo I resulted in pCA-Hpa, while mutagenesis with Oligo II yielded pCA-Hpa (5'Nco). Restriction digestion with *Nco I* and *Hpa I* results in the leader being present (pCA-Hpa) or absent (pCA-Hpa(5'Nco)).

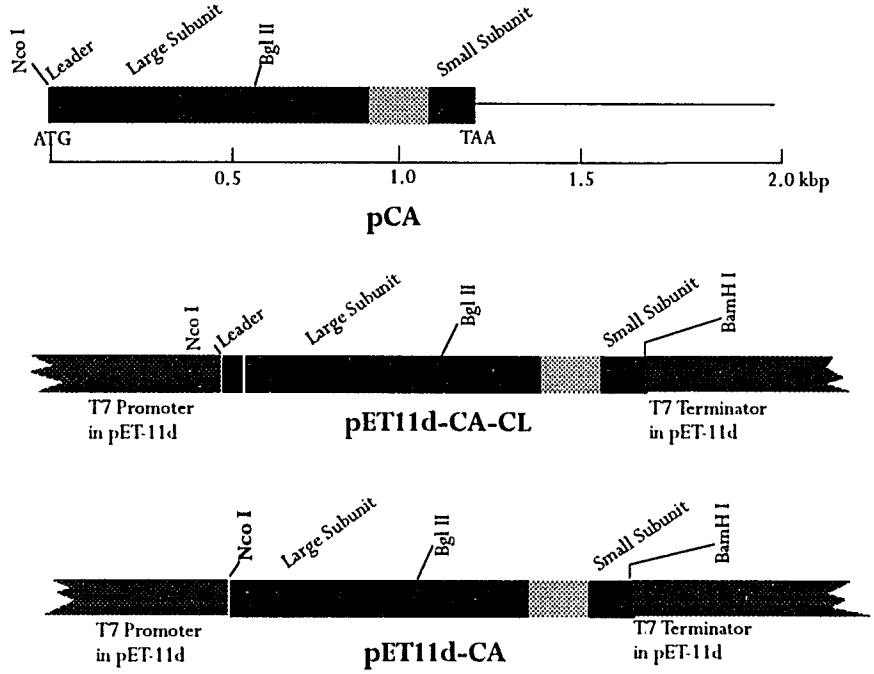

Figure 3. *BamH I* linkers were first added to the blunt ended *Hpa I* 3' ends pCA-Hpa and pCA-Hpa (5'Nco). pET11d-CA-CL was then constructed by cloning the *Nco I* to *BamH I* insert of pCA-Hpa into pET11d, while pET11d-CA was similarly done using the *Nco I* to *BamH I* insert of pCA-Hpa (5'Nco).

removed and the pellet resuspended (vortexed) in 100 µl of 1X SDS protein sample buffer (Sambrook et al. 1989) with 100 mM DTT. The sample was then heated in boiling water for 5 minutes before being subjected (15 µl) to SDS/PAGE analysis.

SDS-PAGE and immunodetection

Protein samples were separated by SDS-PAGE with 15% polyacrylamide, using the Laemmli buffer system (Laemmli, 1970). Gels were stained using colloidal Coomassie brilliant blue G-250 (SIGMA) (Neuhoff et al., 1988). Western blotting was done by transferring separated protein from SDS-PAGE to nitrocellulose at 30V constant voltage for 30 minutes, using the Polyblot transfer system (American Bionetics). The immuno-detection protocol used was described by Geraghty (1989). The primary antibody used in detection, was produced from *C. reinhardtii* peri-CA1, affinity purified using p-aminoethylbenzene-sulfonamide-agarose (SIGMA) (Yang et al., 1985). Polyclonal antibodies were produced in New Zealand rabbits using this antigen as described in Geraghty (1989). The signal of the primary antibody was amplified via biotinylated anti-rabbit IgG as a secondary antibody, then detected using streptavidin-linked horseradish peroxidase with amino ethyl carbazole as the chromogenic substrate.

Carbonic anhydrase activity

Cells from 5 ml of culture 270 minutes after IPTG induction, were collected by centrifugation at 1600 x g for 10 minutes at 4 ^{o}C. The cells were resuspended in 3 ml of ice cold 25 mM barbital, pH 9.25, then sonicated on ice for 30 seconds, three times with one minute intervals to lyse the cells. CA activity was measured at 4 ^{o}C from the pH decrease after the addition of 2 ml of CO_2-saturated water to the bacterial cell lysate in a total volume of 5 ml. Enzyme activity was calculated as: 1 unit = (T_b/T_c)-1, in which T_b and T_c represent times (blank and sample respectively) at 4 ^{o}C needed for a pH change from 8.3 to 7.3 (Spalding and Ogren, 1982).

Protein purification

Two approaches were used for purification of the peri-CA1 protein expressed in *E. coli*. One procedure, described by Geraghty (1989) entailed cutting out the expressed polypeptide from SDS-PAGE of total *E. coli* protein and homogenizing it in buffer (10 mM Tris-HCl, 10 mM EDTA, 5 mM ε-amino-n-caproic acid, 2 mM benzamidine, pH 7.5). This was then agitated overnight at 4 ^{o}C to allow diffusion of protein into the buffer, followed by microcentrifugation. The resulting supernatant was concentrated in a Centricon 30 (AMICON) microconcentrator and used as the purified protein. The second method entailed the purification of protein from inclusion bodies as detailed in Sambrook et al. (1989).

Results

SDS-PAGE analysis of total bacterial protein from cultures with pET11d-CA (Figure 4) shows that a protein of approximate molecular weight (MW) 39 kDa was expressed extensively only in the presence of IPTG. At the time of induction, little of this band was visible. As the time of induction (after IPTG addition) increased however, the amount of this polypeptide also increased. The expressed protein accounted for a significant proportion of total cellular protein after 270 minutes (Figure 4). Western blot analysis, using antiserum derived from affinity purified peri-CA1 antigen from *C. reinhardtii* found that a 39 kDa polypeptide was immunodetected only in the presence of IPTG, as were several smaller polypeptides (Figure 5). Since pET11d-CA does not have the signal sequence, the expected MW of the polypeptide was 39,426 daltons. The abundantly accumulating 39 kDa polypeptide represents the expected size of *C. reinhardtii* peri-CA1 expressed in *E. coli*. The lower MW bands may represent breakdown products of the 39 kDa, that accumulate in the 25 kDa band, which is more resistant to further "rapid" proteolysis (Figure 5).

Induction of pET11d-CA-CL, which includes the native *C. reinhardtii* periplasmic CA target leader (Figures 6 and 7) did not show detectable expression of peri-CA1, using PAGE or polyclonal antibody. Interestingly, when pET11d-CA-CL was induced, growth of the bacteria was inhibited, as measured by A_{600} (Figure 8). Even though the data presented in Figure 8 was obtained from one experiment, this general trend of inhibited

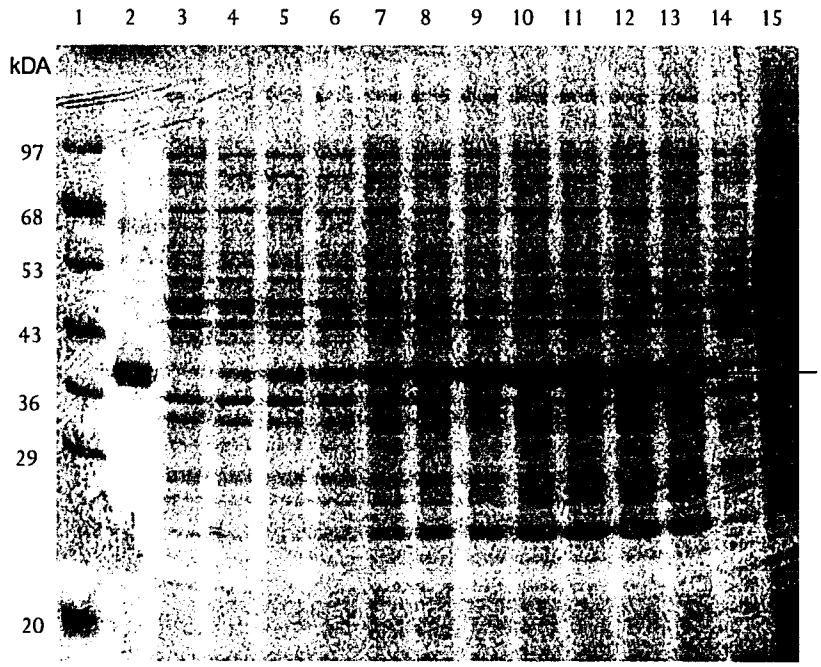

Figure 4. SDS-PAGE showing expression of *C. reinhardtii* periplasmic CA from pET11d-CA in *E. coli*. Lane 1, MW markers; lane 2, purified *C. reinhardtii* periplasmic CA; lane 3, *E. coli* HMS 174 (DE3) with pET11d before IPTG induction; lanes 4 to 13, *E. coli* HMS 174 (DE3) with pET11d-CA at 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes after the addition of IPTG; lane 14, *E. coli* HMS 174 (DE3) with pET11d 270 minutes after IPTG induction; lane 15, *E. coli* HMS 174 (DE3) with pET11d-CA after 270 minutes of growth, with no IPTG added. Arrow indicates 39 kDa peri-CA1 polypeptide of interest.

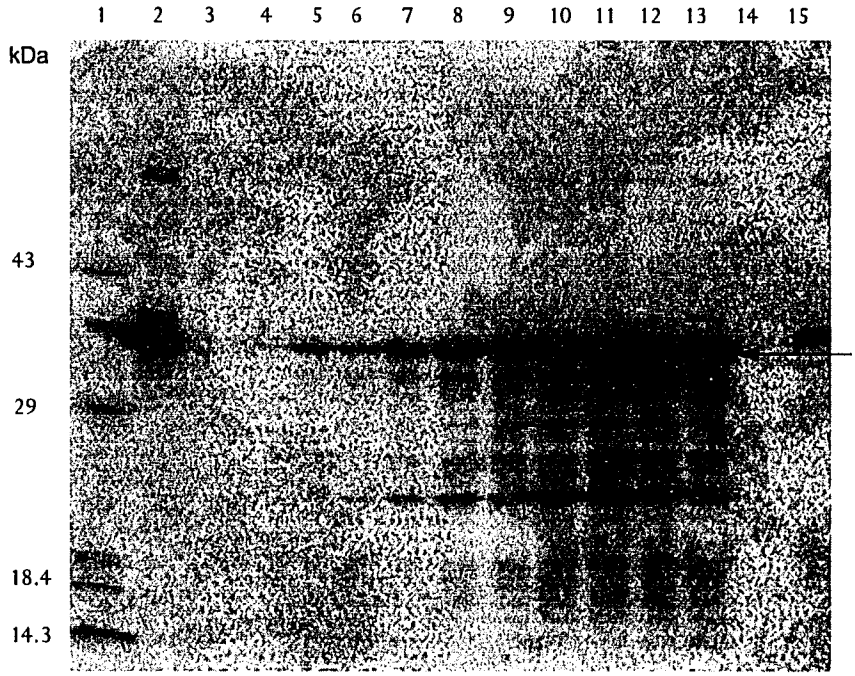

Figure 5. Western blot using affinity purified antiserum generated from *C. reinhardtii* periplasmic CA antigen, showing expression of periplasmic CA from pET11d-CA in *E. coli*. Lane 1, MW markers; lane 2, purified *C. reinhardtii* periplasmic CA; lane 3, *E. coli* HMS 174 (DE3) with pET11d before IPTG induction; lanes 4 to 13, *E. coli* HMS 174 (DE3) with pET11d-CA at 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes after the addition of IPTG; lane 14, *E. coli* HMS 174 (DE3) with pET11d 270 minutes after IPTG induction; lane 15, *E. coli* HMS 174 (DE3) with pET11d-CA after 279 minutes of growth, with no IPTG added. Arrow indicates 39 kDa peri-CA1 polypeptide.

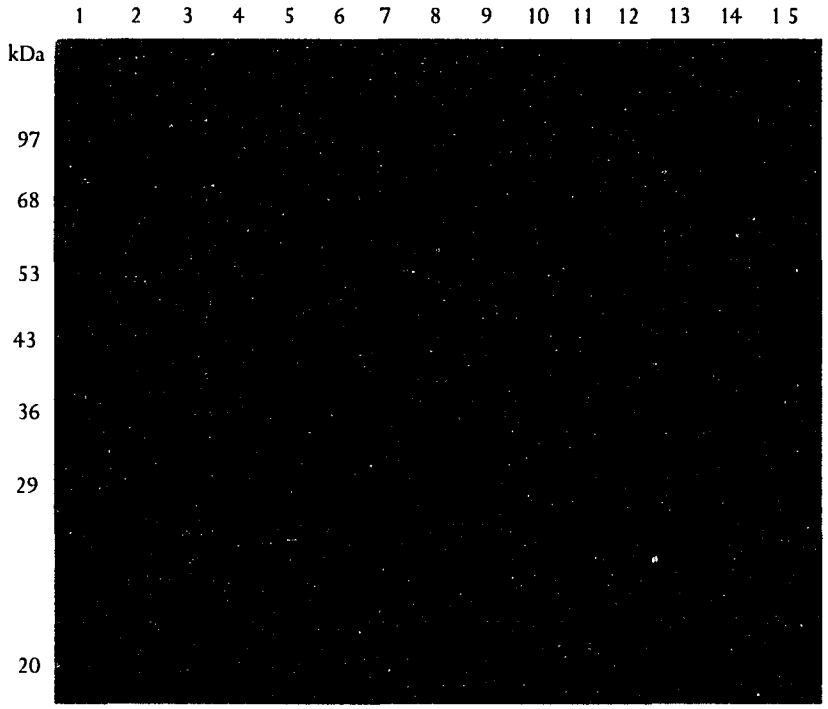

Figure 6. SDS-PAGE showing expression of *C. reinhardtii* periplasmic CA from pET11d-CA-CL in *E. coli* HMS 174 (DE3). Lane 1, MW markers; lane 2, purified *C. reinhardtii* periplasmic CA; lane 3, HMS 174 (DE3) with pET11d before IPTG induction; lanes 4 to 13, *E. coli* with pET11d-CA-CL at 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes after the addition of IPTG; lane 14, *E. coli* with pET11d-CA-CL after 270 minutes of growth, no IPTG added; lane 15, *E. coli* with pET11d 270 minutes after IPTG induction.

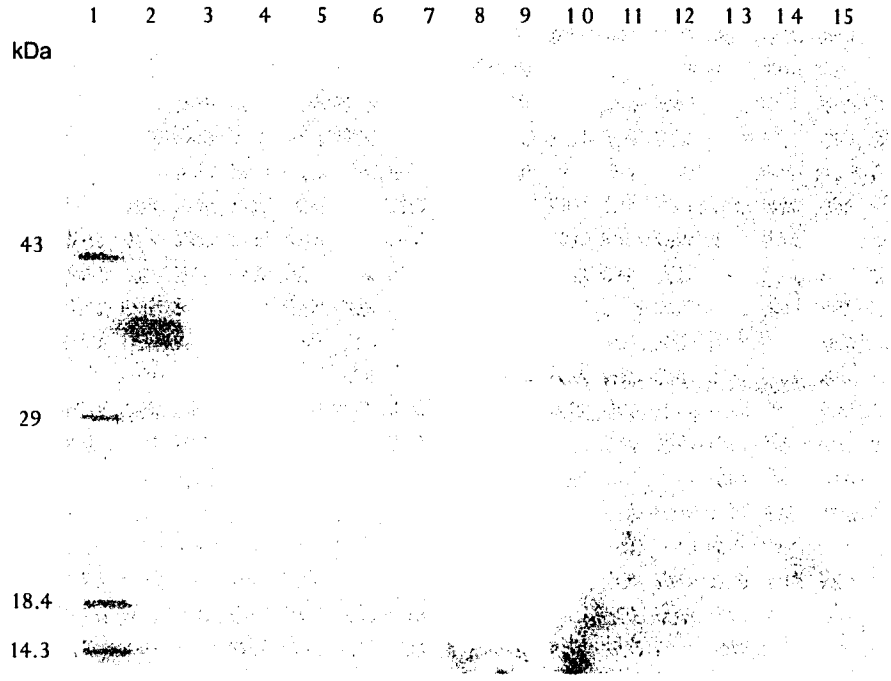

Figure 7. Western blot using affinity purified antiserum generated from *C. reinhardtii* periplasmic CA antigen showing expression of *C. reinhardtii* periplasmic CA from pET11d-CA-CL in *E. coli* HMS 174 (DE3). Lane 1, MW markers; lane 2, purified *C. reinhardtii* periplasmic CA; lane 3, *E. coli* with pET11d before IPTG induction; lanes 4 to 13, *E. coli* with pET11d-CA-CL at 0, 30, 60, 90, 120, 150, 180, 210, 240 and 270 minutes after the addition of IPTG; lane 14, *E. coli* with pET11d-CA-CL after 270 minutes of growth, no IPTG added; lane 15, *E. coli* with pET11d 270 minutes after IPTG induction.

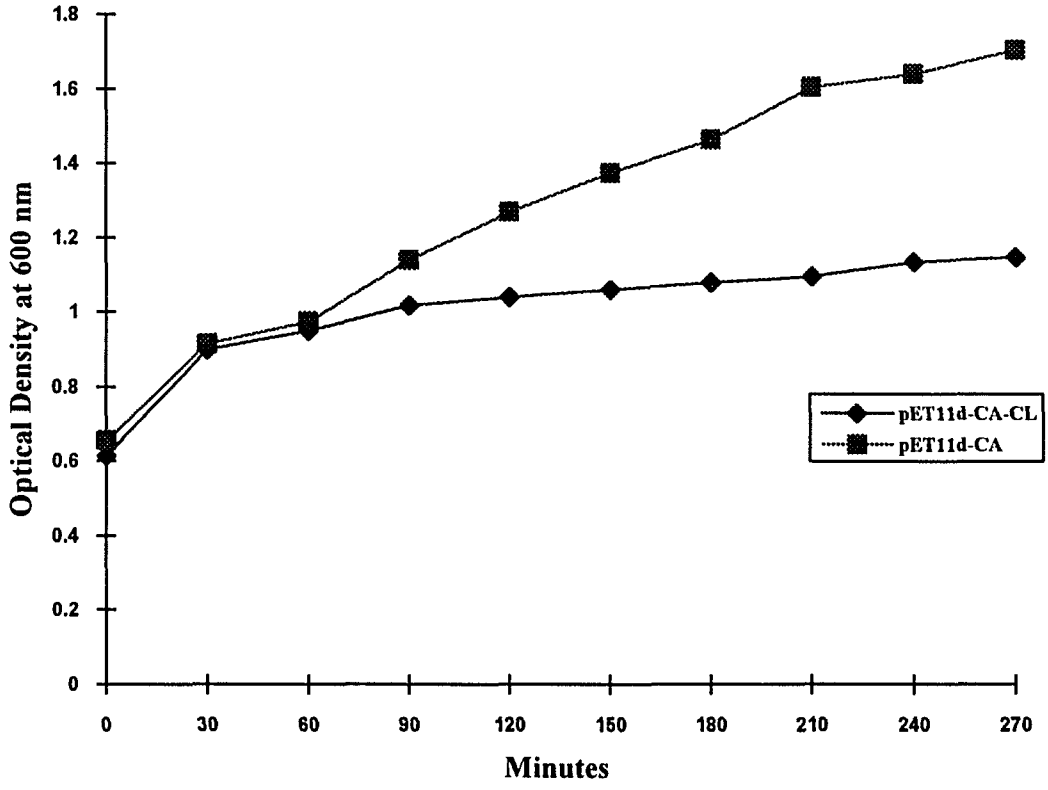

Figure 8. Graph showing time-course of increase (as measured by the optical density at 600 nm) in bacterial cell density of *E. coli* HMS 174 (DE3) with pET11d-CA and pET11d-CA-CL over a 4.5 hour period following induction with IPTG

bacterial growth was consistently observed whenever pET11d-CA-CL was present. When compared to pET11d-CA, pET11d-CA-CL grew similarly for the first hour after induction with IPTG, but with pET11d-CA, bacterial growth continued, while growth when pET11d-CA-CL was present, increased only minimally after the first hour. To further investigate whether the signal sequence in pET11d-CA-CL might be functioning, though making a product at very low levels, cells were collected five hours after IPTG induction, treated to osmotic shock and the periplasmic proteins concentrated. SDS-PAGE, western blot analysis and CA activity assay could not detect the presence of any periplasmic CA protein (data not shown).

Most of the expressed peri-CA1 polypeptide from pET11d-CA was insoluble, apparently forming inclusion bodies as is often observed in *E. coli* overexpression systems. No carbonic anhydrase activity was detected in the crude bacterial cell lysate. Inclusion bodies were pelleted from the lysate, washed, then solubilized in both 8M urea and 6 M guanidine as described in Sambrook et al., (1989). In both instances however, an insoluble precipitate formed when attempts were made to refold the protein. The 39 kDa band was absent when SDS-PAGE of the supernatant was carried out.

The 39 kDa polypeptide as expressed in *E. coli* when pET11d-CA was present, after being identified on SDS-PAGE, was excised from the gel. Gel slices were homogenized in a buffer of 10 mM Tris-HCl, 10 mM EDTA, 5 mM ε-amino-n-caproic acid, 2 mM benzamidine, pH 7.5. The mixture was agitated overnight, thereby allowing the protein in the slices to diffuse out into the buffer. After micro-centrifugation, the protein solution (supernatant) was concentrated, and then used to produce polyclonal antibodies in female New Zealand rabbits as described by Geraghty (1989).

Discussion

This study entailed the expression of *C. reinhardtii* peri-CA1 in *E. coli*, using plasmid constructs with and without the algal gene's target leader sequence, in a T7 RNA/T7 promoter expression system. We obtained very high levels of expression in the bacterial cytoplasm when the leader sequence was absent. The expressed polypeptide which was inactive, insoluble and present in inclusion bodies. No polypeptide could be detected in the bacteria's periplasm or cytoplasm when expression was induced from the construct with signal peptide present. This construct inhibited *E. coli*'s growth

significantly one hour after induction. Successful expression of peri-CA1 in *E. coli* allowed us to have adequate amounts of protein free of oligosaccharides to serve as antigen for polyclonal antibody production.

There was a band at approximately 25 kDa that also accumulated, albeit at lower level than the 39 kDa band, as time of incubation increased (Figure 4). This 25 kDa band also cross-reacted with anti-periplasmic CA (Figure 5). One can speculate that the 39 kDa product may slowly degrade, with the 25 kDa band representing a fairly stable intermediate, somewhat resistant to further proteolytic degradation. The antiserum detected numerous bands intermediate in size between these two bands (Figure 5), and to a significantly lesser extent, below the 25 kDa band, which would support the above speculation.

The band in lane 15 of Figures 5, at around 39 to 40 kDa that cross-reacted with the antibody is more difficult to explain. pET11d-CA was not IPTG induced in this lane. It is however possible that the *lac* repressor was not 100 % effective, and over time (lane 15 represents 4.5 hours of further incubation after A_{600} was reached) some "spontaneous" induction occurred.

Attempts to express the full-length protein, including the target peptide, using pET11d-CA-CL did not result in the detectable expression of a product, either by protein staining or immunoblot, (Figures 6 and 7). In fact this construct apparently had a negative effect on bacterial growth (Figure 8), causing substantial decrease in growth 1 to 1.5 hours after IPTG induction. Based on the growth inhibition, one is tempted to surmise that the full-length protein was expressed at a level that was not detectable, but was sufficient to be detrimental to survival or continued growth of the *E. coli*. Assuming this to be true, it is possible that inclusion of the leader sequence resulted in the peri-CA1 becoming anchored to the inner membrane of the bacteria by the relatively hydrophobic targeting peptide (Figure 9). Such anchoring would disrupt the membrane, making it leaky or disrupting the function of other membrane proteins.

There was abundant protein expression from pET11d-CA, however, this protein was insoluble, and apparently present in inclusion bodies. Inclusion bodies, which are dense aggregates of insoluble protein and RNA, are generally formed following high level expression of foreign proteins and contain most of the foreign (Schein, 1989). In *Chlamydomonas*, peri-CA1 is targeted to the ER, where it is glycosylated. The mature protein results after proteolytic cleavage, yielding a large and small subunits, held together by disulfide bonds in a heterotetramer. The generation of inclusion bodies might

also have resulted in part, from the expressed protein assuming an aberrant folding pattern due to the lack of proper post-translational processing in E. coli. Attempts to refold the protein in a soluble form following solubilization of the inclusion bodies with chaotropic agents were unsuccessful.

In conclusion, we were successful in expressing *C. reinhardtii* periplasmic CA in the cytoplasm of *E. coli*. Even though it was insoluble, the protein accumulated to very high levels, readily allowing us to have adequate quantities for antibody production. When the algal target sequence was present, expression could not be detected, nonetheless, bacterial growth was drastically inhibited.

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Figure 9. Amino acid sequence of *Chlamydomonas reinhardtii* periplasmic CA signal leader showing basic, neutral and hydrophobic residues. -1 is the first residue before the cleavage site.

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EXPRESSION OF *Chlamydomonas reinhardtii* PERIPLASMIC CARBONIC ANHYDRASE IN TRANSGENIC TOBACCO PLANTS

A paper to be submitted to the Plant Molecular Biology journal

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Abstract

The periplasmic carbonic anhydrase (CA) gene *CAH1* of *Chlamydomonas reinhardtii* codes for a highly processed secreted glycoprotein. The primary translation product of the *CAH1* gene is targeted to the ER, where it is proteolytically processed to yield two different subunits, glycosylated, assembled into an active heterotetramer, and secreted. After replacing the target leader sequence with that from tobacco anionic peroxidase, expression of this gene in transgenic tobacco plants was investigated. Of several plants screened by immunoblotting and found to express periplasmic CA 1 (peri-CA1), a single plant, TL_1, with a high level of expression was chosen for further analysis. SDS-PAGE gels of the purified protein from tobacco, showed that it migrated as a series of discrete bands (two large and one small) with slightly faster mobility than the comparable bands in the purified algal protein. The expressed protein in the plant was active, and staining with thymol and sulfuric acid confirmed that it was also glycosylated. Analysis of vacuum infiltrates from leaves expressing peri-CA1 showed that it was enriched in the intercellular fluid. The sensitivity of the enzyme to sulfonamide inhibitors was similar to that of the native algal enzyme. These results suggest that the post translational processing of *Chlamydomonas* periplasmic CA 1 is largely conserved in a higher plant.

Introduction

Carbonic anhydrase (CA) has been found in several higher plants. It catalyses the hydration-dehydration reaction:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

The molecular weight (MW) of the enzyme in dicotyledonous plants ranges from 140 kDa to 250 kDa, comprised usually of 6 subunits, each around 30 kDa (Graham et al., 1984). In monocotyledons, the protein is of a low MW, around 42 to 45 kDa, with a subunit of 27.5 kDa (Atkins et al., 1972a). There is one atom of zinc associated with each subunit, and the enzyme is less sensitive to sulfonamides than mammalian CA (Atkins et al., 1972b). In C_3 plants, CA is an abundant protein, predominantly located in the stroma of chloroplasts, (Jacobson et al., 1975; Tsuzuki et al., 1985), even though cytosolic forms have been suggested (Reed and Graham, 1981; Graham et al., 1984). CA of C_4 leaves is almost exclusively localized to the cytosol of the mesophyll cells (Burnell and Hatch, 1988). Immunological studies show some cross-reactivity between CA originating from C_3 and C_4 species of dicot and monocot (Burnell, 1990). Fukuzawa et al. (1992) reported that chloroplast CAs from C_3 plants show a high degree of sequence similarity (approximately 76%) to each other, and that cytosolic CA from maize had a 60% sequence similarity to chloroplast CAs.

A precise role for CA in C_3 plants is not clear, though it is speculated to be involved in photosynthesis. It has been proposed that CA in the alkaline stroma of the chloroplast aids the transport of inorganic carbon (C_i) across the chloroplast by facilitated diffusion, (Raven and Glidewell, 1978; Cowan, 1986; Badger and Price, 1994), thereby minimizing the gradient in CO_2 concentration between the chloroplast envelope and Rubisco. It has also been suggested that CA may play a role in CO_2 fixation due to special association between CA and Rubisco which allows for elevated CO_2 at the active site of Rubisco (Bird et al., 1980; Reed and Graham, 1981; Majeau and Coleman, 1994; Badger and Price, 1994). In C_4 plants CA converts CO_2 entering the mesophyll cells to HCO_3^-, the substrate for phosphoenolpyruvate carboxylase, in the first step in C_4 photosynthesis (Hatch and Burnell, 1990).

Chlamydomonas reinhardtii is a unicellular, eukaryotic, green alga. Studies have indicated that CA activity exists in the algae's cytosol, chloroplast (Moroney et al., 1987; Husic et al., 1989; Sultemeyer et al., 1990), and periplasm (Kimpel et al., 1983; Coleman et al., 1984b; Yang et al., 1985). Induction of these different forms of CA occurs when *C. reinhardtii* cells are transferred from growth at high CO_2 to air (Badger and Price, 1994). There are however two different periplasmic CAs, *CAH1* and *CAH2*. and the latter is

down-regulated by limiting external C_i (Fujiwara et al., 1990). At air levels of CO_2, an active CO_2 concentrating mechanism (CCM) is induced, whereby CO_2 can be concentrated internally 40-fold higher than exists in the media (Badger et al., 1980). The periplasmic *CAH1* form of CA is expressed at very high levels during the CCM, accounting for 80 to 95% of CA activity (Kimpel et al., 1983; Coleman et al., 1984; Yang et al., 1985). The precise role of this periplasmic CA in the CCM is not clear, although it probably converts external HCO_3^- to CO_2, which can be taken up through the plasma membrane and then be used in photosynthesis (Badger and Price, 1994).

The two periplasmic CA genes, *CAH1* and *CAH2*, show 92% sequence homology (Fujiwara et al., 1990; Fukuzawa et al., 1990). *C. reinhardtii* periplasmic CAs show greater homology with mammalian CAs (especially around the active site) than with plant chloroplast CAs (Fujiwara et al., 1990; Fukuzawa et al., 1990). It would appear that plant chloroplast CAs may have evolved from a common ancestor, which is however of a different source compared to *C. reinhardtii* periplasmic CAs and mammalian CAs (Fukuzawa et al., 1992).

Protein secretion is a multi-step process in eukaryotes, entailing translocation across the endoplasmic reticulum (ER) which requires a signal sequence on the protein, transport to the Golgi complex, and vesicle mediated delivery at the plasma membrane (Walter and Lingappa, 1986). Many polypeptides entering in the ER lumen are N-glycosylated, i.e. oligosaccharides are added at asparagine residues present in the sequence Asn-Xaa-Ser/Thr, where Xaa is any amino acid other than proline. Protein disulfide isomerase may also catalyze the formation of disulfide bonds in the oxidizing environment of the ER (reviewed by Vitale et al., 1993). Castor bean ricin (Butterworth and Lord, 1983; Halling et al., 1985) and legumin of legume seeds (Lycett et al., 1984) are synthesized as precursors, then cleaved into two subunits which remain linked by disulfide bonds . Finally, it is generally accepted that in yeasts and mammals, specific sorting signals exist which are recognized in the Golgi, that target proteins to vacuoles, lysosomes or re-entry to the ER (Munro and Pelham, 1987; Pelham et al., 1988). Removal of such signals result in "default" secretion of protein. There is also regulated secretion of proteins where release is mediated by extracellular signals (Burgess and Kelly, 1987). It is not clear whether final secretion from the plant cell is via a default pathway or if specific secretory signals exists on the proteins.

CAH1 is a nuclear encoded gene from which the secreted protein, periplasmic CA 1 (peri-CA1) is synthesized as a precursor of 41.6 kDa polypeptide, which is processed

post-translationally to a glycosylated 38 kDa large subunit and 4.2 kDa small subunit (Toguri et al., 1986; Fukuzawa et al., 1990; Kamo et al., 1990; Coleman, 1991). The precursor has an N-terminal 20-amino-acid signal sequence that targets the polypeptide to the endoplasmic reticulum (Fukuzawa et al., 1990). During processing, a 35 amino acid peptide between the large and small subunit is proteolytically deleted, while oligomerization results in a heterotetramer protein held together with disulfide bonds (Kamo et al., 1990; Ishida et al., 1993). There are also three asparagine-linked N-glycosylation sites, Asn-Xaa-Thr/Ser, in the large subunit which are all glycosylated (Ishida et al., 1993). On SDS PAGE the large subunits appears as a doublet of bands, 35 to 37 kDa suggesting differential glycosylation, as deglycosylation yields a single polypeptide of 32 kDa (Toguri et al., 1986).

The work reported here was carried out to assess the expression, in terms of activity, assembly, and targeting of *Chlamydomonas* peri-CA1 to the apoplast of transgenic tobacco. Further, because of the extensive post-translational processing of peri-CA1 that occurs in *C. reinhardtii,* it was of interest to evaluate how much of this would be conserved by the higher plant.

Materials and methods

Plasmid construction

A full length cDNA clone of *CAH1*, designated pCA, was obtained by taking advantage of the published sequence by Fukuzawa et al (1990) to extend a partial cDNA clone (Spalding et al., 1991) to full length using PCR (Winder, 1991). To facilitate use of the *Nco I* site at the translation start site (+1) in vector construction, the *Nco I* site at position +123 was eliminated by changing His^{42} codon from CAT to CAC (Winder, 1991). Using the site-directed mutagenesis method of Kunkel et al. (1985), a *Hpa I* site was added at +1138 after the stop codon by changing the "C" at +1136 to a "G", and "CC" at +1139, +1140 to "AA" (see Figure 1). This construct was designated pCA-Hpa. Next an *Nco I* site was introduced at +53, between the leader sequence and the large subunit. This resulted in Ala^{18} GCG being changed to GCC, and Gln^{19} codon CAG being replaced with Met codon ATG. This second construct was designated pCA-Hpa (5'Nco) (Figure 2). A 26-mer (Oligo-1) spanning +1125 to +1150 was used to effect

```
                                                    GAGTCATTACCTGCAACCCACTTGAACACC   -1
                                                                          CAT
+1  ATGGCGCGTACTGGCGCTCTACTCCTGGTCGCGCTGGCGCTTGCGGGCTGCGCGCAGGCT                  60

61  TGCATCTACAAGTTCGGCACGTCGCCGGACTCCAAGGCCACCGTTTCGGGTGATCACTGG                  120
        C
121 GACCATGGCCTCAACGGCGAGAACTGGGAGGGCAAGGACGGCGCAGGCAACGCCTGGGTT                  180

181 TGCAAGACTGGCCGCAAGCAGTCGCCCATCAACGTGCCCCAGTACCAGGTCCTGGACGGG                  240

241 AAGGGTTCCAAGATTGCCAACGGCCTGCAGACCCAGTGGTCGTACCCTGACCTGATGTCC                  300

301 AACGGCACCTCGGTCCAAGTCATCAACAACGGCCACACCATCCAGGTGCAGTGGACTTAC                  360

361 AACTACGCCGGCCATGCCACCATCGCCATCCCTGCCATGCACAACCAGACCAACCGCATC                  420

421 GTGGACGTGCTGGAGATGCGCCCCAACGACGCCGCCGACCGCGTGACTGCCGTGCCCACC                  480

481 CAGTTCCACTTCCACTCCACCTCGGAGCACCTGCTGGCGGGCAAGATCTATCCCCTTGAG                  540

541 TTGCACATTGTGCACCAGGTGACTGAGAAGCTGGAGGCCTGCAAGGGCGGCTGCTTCAGC                  600

601 GTCACCGGCATCCTGTTCCAGCTCGACAACGGCCCCGATAACGAGCTGCTTGAGCCCATC                  660

661 TTTGCGAACATGCCCTCGCGCGAGGGCACCTTCAGCAACCTGCCGGCGGGCACCACCATC                  720

721 AAGCTGGGTGAGCTGCTGCCCAGCGACCGCGACTACGTAACGTACGAGGGCAGCCTCACC                  780

781 ACCCCGCCCTGCAGCGAGGGCCTGCTGTGGCACGTCATGACCCAGCCGCAGCGCATCAGC                  840

841 TTCGGCCAGTGGAACCGCTACCGCCTGGCTGTGGGCCTGAAGGAGTGCAACTCCACGGAG                  900

901 ACCGCCGCGGACGCCGGCCACCACCACCACCACCGCCGCCTGCTGCACAACCACGCGCAC                  960

961 CTGGAGGAGGTGCCTGCCGCCACCTCCGAGCCCAAGCACTACTTCCGCCGCGTGATGCTG                 1020

1021 GCCGAGTCCGCGAACCCCGATGCCTACACCTGCAAGGCCGTTGCCTTTGGCCAGAACTTC                 1080
                                                                  G   AA
1081 CGCAACCCCCAGTACGCCAACGGCCGCACCATCAAGCTGGCCCGCTATCACTAAACTTCC               1140

1141 CAGTAGTTAGTCACGCTACCACCGTCGGCACGGCCAGCAGGCATTCCATTTTCCAGGCTT                 1200

1201 TGCTTCACGGTTTGGTGTGTCATTCGATGGTGTTCTTGACGACCCGCGCTTGGCGGGCCT                 1260
1261 TTCCAATTTTTTCCATAGTACACCGAAATAGTTCTGCGGTGCAGCACGCATACACACAGT                 1320
1321 ACCGGACGGGCGGCGGGACCTCCTGTTTTCTCCTGACTAGTAAAGAAGTAAGGAAGGTAT                 1380
1381 GGAGTTGGTTCCACGATGGGGCAGTCTGAGAGCGGAATAAAGTCAGTGGGCCGGACGTTG                 1440
1441 TGGCGATGGATGGTAGTGAGGCAAGTAATACGTACGTAGAGGGCGTACGCGGGTAATAAC                 1500
1501 GGGAACTTCGACAGCAATCGAGAGTGTCTGCACGCGAGACATTTGCGTACAGGGGAGGCA                 1560
1561 CCGGTTCTCCTCGATGAGTGATCCGTACTTATGCAAGTTATATAAGGCTGGTGTGGGGCG                 1620
1621 CTTCAGCACGGTATGGTTGCCAGCATGCACGGTCCGGCCTCTGTCTGGCTGGCTGGGTTG                 1680
1681 TTGGCGGGCTGGCCTCATGCGCGCCGTCGCACATGCCGATCAATGCAGTTGCTCTCCAGT                 1740
1741 AGCTGCAAGGCCTGGCTGGGCAATCCCATAGCCATGTCGAATGTGAAGCATTGTTTTCTT                 1800
1801 GGAGATGGAGGACAGGAGACGCTGACCGGATGTTTTAAGACGTGCAGGATGTGGGGAGCG                 1860
1861 AGGTAGCTACAACGGTGCAGTTGAGGCAGAGACGTGTACGACATGTAAGATGCCCATGGA                 1920
1921 CAAAAAAAAAAAAAAAAAAAA                                                       1942
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Figure 1. cDNA nucleotide sequence of *Chlamydomonas reinhardtii* periplasmic CA showing bases changed (**bold-face**), new bases substituted (*italic*) via site directed mutagenesis, along with Oligo I and Oligo II used (underlined).

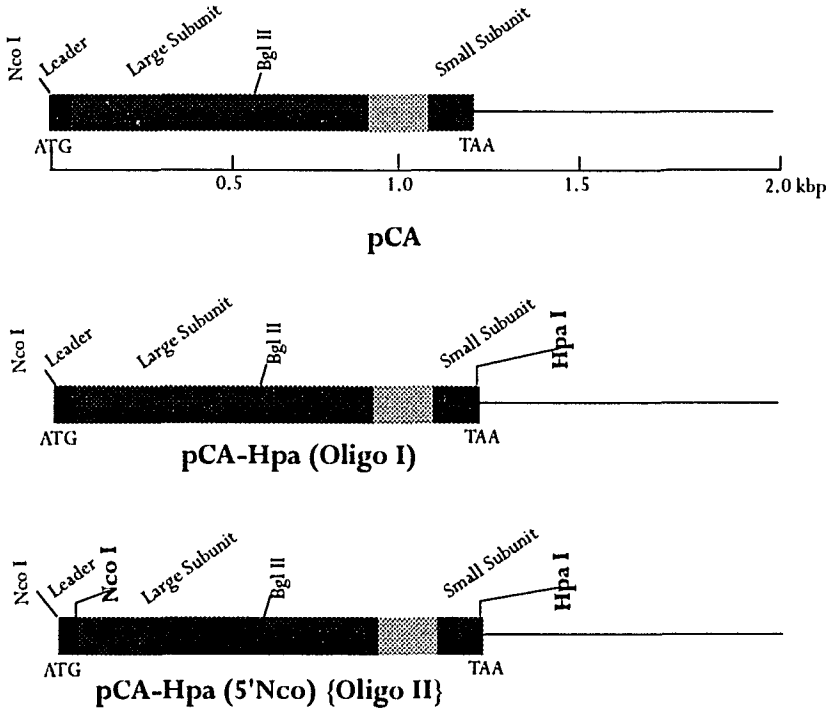

Figure 2. Site directed mutagenesis of pCA with Oligo I resulted in pCA-Hpa, while mutagenesis with Oligo II yielded pCA-Hpa (5'Nco). Restriction digestion with *Nco I* and *Hpa I* results in the leader being present (pCA-Hpa) or absent (pCA-Hpa(5'Nco)).

mutagenesis in the former, while a 27-mer (Oligo-2) spanning +43 to +69 was used in the latter (Figure 1).

pCA-Hpa (5'Nco) (no leader) was first cut with *Hpa I*, then *BamH I* linkers ligated to the blunt ends (Sambrook et al. 1989), followed by cutting with *Nco I* and *BamH I*. The resulting open reading frame insert with *Nco I* 5' and *BamH I* 3' ends was then ligated with the *Nco I/BamH I* ends of the *Escherichia coli* expression vector pET11d (Novagen), which carries the bacteriophage T7 ϕ10 promoter and ϕ Terminator. The completed plasmid was designated pET11d-CA (no leader) (see Figure 3). This construct was used to transform bacterial host HMS174 (DE3), an *E. coli* K12 strain (F^- *recA* $r_{k12} - m_{k12}+$ Rif^r) lysogenized with a lambda derivative, DE3. DE3 carries the immunity region of phage 21, the *lacI* gene, the *lac*UV5 promoter, the start of the *lacZ* gene and the gene for T7 polymerase inserted into the *int* region of the phage (Studier and Moffat, 1986). The *lac*UV5 promoter directs transcription of T7 RNA polymerase upon the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Studier et al., 1990).

pCA-Hpa (5'Nco) (no periplasmic CA leader) was cut with *Hpa I* and *Nco I*, resulting in a open reading frame insert with *Nco I* and *Hpa I* ends. To this open reading frame of the *CAH1* gene was ligated a *Bgl II/Nco I* cauliflower mosaic virus (CaMV) promoter cloned from pPHP425 and a *Hpa I/Eco RI* PIN II terminator fragment from pPHP291. The vectors pPHP425 and pPHP291 were gifts from G. Huffman, Pioneer Hybrid International. The "promoter" sequence included a duplicated upstream segment of the 35S promoter from CaMV (Kay et al., 1987), the untranslated leader sequence Ω' of tobacco mosaic virus RNA which serves as a translational enhancer (Gallie et al. 1987, Gallie and Kado, 1989) and the tobacco peroxidase leader sequence (Lagrimini et al., 1987) for targeting to the ER. This entire *Bgl II - Eco RI* fragment was then cloned into the binary vector pBin19 (Bevan, 1984), suitable for *Agrobacterium tumefaciens* mediated transformation of plant cells and designated pBin-TL-CA (see Figure 4). Protocols for DNA cloning were according to Sambrook et al., (1989).

Antibody production

C. reinhardtii peri-CA1 was expressed in *E. coli*. The expressed protein was identified on SDS-PAGE, the band excised and homogenized in buffer (10 mM

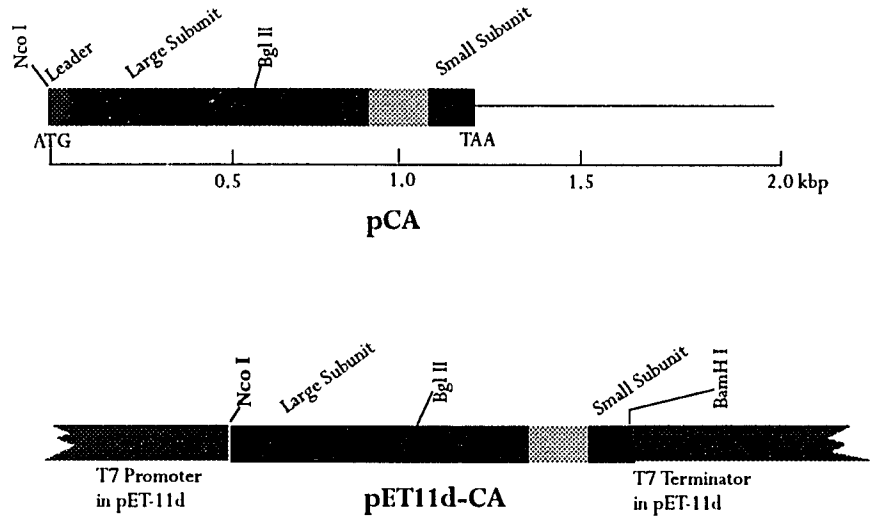

Figure 3. *BamH I* linkers were first added to the blunt ended *Hpa I* 3' ends pCA-Hpa (5'Nco). pET11d-CA was then constructed by cloning the *Nco I* to *BamH I* insert of pCA-Hpa into pET11d.

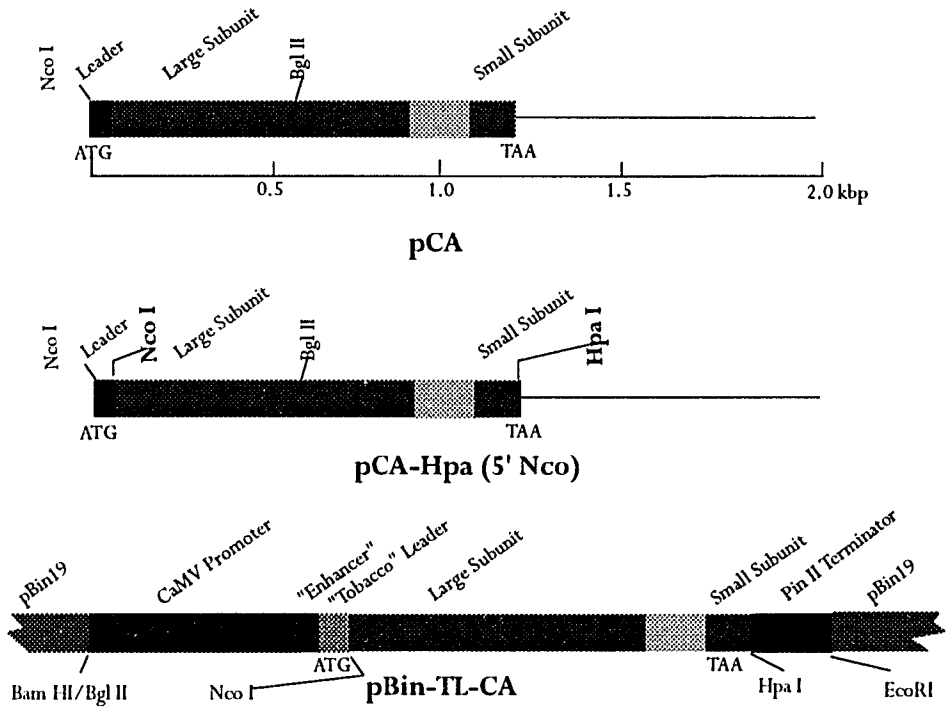

Figure 4. Site directed mutagenesis of pCA to introduce *Nco I* and *Hpa I* sites resulted in pCA-Hpa (5'Nco). Restriction with *Nco I* and *Hpa I*, then ligating Pin II terminator and CaMV promoter, followed by cloning into pBin19 yielded pBin-TL-CA.

Tris-HCl, 10 mM EDTA, 5 mM ε-amino-n-caproic acid, 2 mM benzamidine, pH 7.5). This mixture was agitated overnight at 4 ^{o}C, facilitating diffusion of the protein into the buffer. The mixture was then centrifuged and the supernatant concentrated at 4 ^{o}C using a Centricon 30 (AMICON). This purified protein was used to produce polyclonal antibodies in female New Zealand rabbits as described by Geraghty (1989).

The antiserum was purified via affinity chromatography. *C. reinhardtii* peri-CA1 was purified using p-aminoethylbenzene-sulfonamide-agarose (SIGMA) according to the previously reported method of Yang et al., (1985) This protein was then bound to CNBr-activated of Sepharose CL 4B agarose (SIGMA). The protein-Sepharose conjugate (3 ml) was next added to a column (50 x 1 cm) containing 30 ml of Sephadex G-25. The bacterial antigen antiserum was then poured onto this column, and was allowed to set for 1 hour, followed by the unbound antisera being washed away with excess PBS buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4). The bound antibodies were then eluted using 3 ml of 3 M sodium thiocyanate, this chased immediately with excess PBS. The desired antibody front was monitored (280 nm), collected, concentrated in a Centricon 30 (AMICON) and used as purified *C. reinhardtii* peri-CA1 antibody.

Expression in *E. coli*

Expression was carried out as described by Sano and Cantor (1990) with a few modifications. The HMS174(DE3) bacterial host carrying the target plasmid pET11d-CA was grown in a 50 ml culture at 37^{o} C with shaking in 2X YT medium (Sambrook et al. 1989) supplemented with 50 μg/ml ampicillin. When the A_{600} reached 0.6, a 1 ml sample was taken before adding IPTG to a final concentration 1 mM to induce the T7 RNA polymerase gene under the *lac*UV5 promoter. The culture was allowed to grow a further five hours, with 1 ml samples being taken for analysis of heterologous protein at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 hours after IPTG induction. Cells from 1 ml of the above culture were microcentrifuged for 60 seconds at 4^{o} C. The supernatant was removed and the pellet resuspended (vortexed) in 100 μl of 1X SDS protein sample buffer (Sambrook et al. 1989) with 100 mM DTT. The sample was then heated in boiling water for 5 minutes before being subjected (15 μl) to SDS/PAGE analysis.

Production of transgenic tobacco plants

pBin-TL-CA was electroporated into *A. tumefaciens* strain LBA4404, with transformants being detected via growth on YEP agar (Sambrook et al., 1989). Transformation of tobacco (*Nicotiana tabacum, cv* Xanthi) was effected by cocultivating leaves of sterile plants, cut into small pieces (0.5 x 0.5 cm^2), for two days in a 1:10 dilution of an overnight culture of the transfected *Agrobacterium* containing pBin-TL-CA, as described previously (An et al., 1986). Subsequently, regenerated plantlets were grown at 28^{o}C under 3000 lux light for 12 hour day and 12 hour night for three weeks. Regenerated plants were transferred to soil in small pots, in covered containers held at 28 ^{o}C. They were gradually opened over a seven day period, after which time the fully acclimatized plants were transferred to the greenhouse.

Preparation of protein extracts

Interveinal leaf tissue from one leaf was excised and cut lengthwise into strips about 0.5 cm wide. Half of the strips (0.3 g) were frozen in liquid nitrogen, then ground in a cold mortar and pestle, followed by suspension in 600 µl of ice-cold extraction buffer (100 mM Tris-HCl, pH 7.8, 500 mM sucrose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM ε-amino-n-caproic acid, 2 mM benzamidine, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM ascorbic acid and 1.5 % polyvinylpolypyrrolidone (PVPP)). The mixture was vortexed, then microcentrifuged for 15 minutes, and the supernatant removed, followed by re-microcentrifugation for 10 minutes. This supernatant was then collected and designated the "total extract". The remaining leaf strips (0.3 g) were immersed in ice-cold extraction buffer (less the PVPP), and vacuum infiltrated for three periods of one minute each. The pieces were then gently blotted dry, rolled up and placed in a 15 ml tube from which bottom was cut off in a manner, such that the leaf pieces were retained during centrifugation. This was then placed in 50 ml conical bottom tube and centrifuged at 3000 rpm, using a SS34 rotor, for 10 minutes at 4 ^{o}C. The fluid that collected in the conical bottom tube was designated "intercellular fluid" (IF). The tissue remaining after centrifugation was treated the same way as the total extract and was designated "residual cellular extract". All samples were then adjusted to 800 µl each. Periplasmic CA was purified from the protein extracts by affinity chromatography on

immobilized p-aminoethylbenzene-sulfonamide-agarose (SIGMA) column using the method described by Yang et al., (1985). Preparation of leaf protein extract for "plant leaf CA assay" was as described by Hatch (1991). Protein concentration on all samples was determined using the commercial Bio-Rad dye-binding protein assay kit. Bovine serum albumin was used as the protein standard.

SDS-PAGE and immunoblots

Protein samples were separated by SDS-PAGE with 15% polyacrylamide, using the Laemmli buffer system (Laemmli, 1970). Gels were stained using colloidal Coomassie brilliant blue G-250 (SIGMA) (Neuhoff et al., 1988). Western blotting was done by transferring separated protein from SDS-PAGE to nitrocellulose at 30V constant voltage for 30 minutes, using the Polyblot transfer system (American Bionetics). The immunodetection protocol used was described by Geraghty (1989). Affinity purified anti-peri-CA1 polyclonal antibody was the primary antibody used in detection. The signal of the primary antibody was amplified via biotinylated anti-rabbit IgG as a secondary antibody, then detected using streptavidin-linked horseradish peroxidase with amino ethyl carbazole as the chromogenic substrate.

Subunit constitution was ascertained using SDS-PAGE with 15% polyacrylamide, using the Laemmli buffer system (Laemmli, 1970). After electrophoresis the gels were fixed with 40% methanol/7% acetic acid/1% glutaraldehyde. Staining was as previously described using colloidal Coomassie brilliant blue G-250.

Glycoproteins were detected in polyacrylamide gels using thymol and sulfuric acid (Racusen, 1979; Gerard, 1990). After SDS-PAGE, the gel was fixed in 25% 2-propanol/10% acetic acid, then immersed in this same solution containing 0.2% thymol for 90 minutes. Glycoproteins appeared as pink bands after immersion in 80% sulfuric acid/20% ethanol. After soaking overnight in 10% acetic acid/10% (v/v) methanol, the gel was counterstained with colloidal Coomassie brilliant blue G-250.

RNA extraction and analysis

Total RNA was isolated from untransformed and transgenic leaf tissue, ground in liquid nitrogen with a mortar and pestle. The method used was that of Wadsworth et al., (1988) as modified by Seeley et al., (1992). Samples containing 20 μg of total RNA were resolved on a denaturing 2% agarose/6% formaldehyde gel and blotted onto a nitrocellulose filter (Hybond-N, Amersham). Following UV cross-linking, the nitrocellulose filters were hybridized with a ^{32}P random-primer-labeled (Foinberg and Vogelstein, 1984) *Nco I-BglII* fragment of pCA cDNA. Autoradiography was performed using X-omat AR film (Kodak) at -70 °C with an intensifying screen.

Activity assays

Peri-CA1 activity was assayed by adding 20 μl of protein extract in 3 ml of ice cold 25 mM barbital, pH 9.25 and mixing at 4 °C. 10 mM dithiothreitol was added to this mixture when "plant CA" was being assayed. Two ml of CO_2-saturated water was then added to the barbital mixture and the pH decrease measured. Enzyme activity was calculated as: 1 unit = (T_b/T_c)-1, in which T_b and T_c represent times (blank and sample respectively) at 4 °C needed for a pH change from 8.3 to 7.3 (Spalding and Ogren, 1982). Inhibition of CA activity by acetozolamide and ethoxyzolamide was carried out according to a method described by Husic et al., (1989).

The peroxidase activity assay was adapted from Rathmell and Sequeira (1974). Twenty μl of extract (IF, cell material or total protein) was mixed with 965 μl of 25 mM sodium acetate buffer, pH 4.5 and 15 μl of 20 mM guaiacol (SIGMA). The reaction was started by adding 4 μl of 40 mM H_2O_2 and measuring absorbance at 470 nm. One unit of enzyme is the amount causing an absorbance increase of 1.0/min at 24 °C.

The NAD-dependent malic dehydrogenase assay was adapted from Edwards and Gutierrez (1972). 500 μl of 100 mM Tricine, pH 8.0, 50 μl 100 mM $MgCl_2$, 15 μl 10 mM NADH, 415 μl ddH_2O and 10 μl of protein extract were mixed in a quartz cuvette. 15 μl 200 mM oxaloacetic acid was then mixed quickly to start the reaction. The decrease in absorbance at 340 nm was measured. One enzyme unit is defined as the number of μ

moles of NADH oxidized per min at 25 °C. Specific activity was the number of enzyme units per mg protein.

Results

Expression of *Chlamydomonas* periplasmic CA in *E. coli*

SDS-PAGE analysis of total bacterial protein from cultures with pET11d-CA (Figure 5) shows that a protein of approximate MW 39,000 was expressed extensively only in the presence of IPTG. At the time of induction, little of this band is visible. As the time of induction (after IPTG addition) increased however, the amount of this polypeptide also increased. Since pET11d-CA does not have the signal sequence, the expected MW of the polypeptide was 39,426 daltons. The expressed protein accounted for a very significant percent of total cellular protein after 4.5 hours (Figure 5). Western blot analysis, using antiserum derived from purified peri-CA1 antigen from *C. reinhardtii* found that a 39 kDa polypeptide was immunodetected only in the presence of IPTG, as were several smaller polypeptides (Figure 6). The abundantly accumulating 39 kDa polypeptide represents the expected size of *C. reinhardtii* peri-CA1 expressed in *E. coli*. The lower MW bands may represent breakdown products of the 39 kDa, that accumulate in the 25 kDa band, which is resistant to further proteolysis (Figures 5 and 6).

Expression of *Chlamydomonas* periplasmic CA in transgenic plants

C. reinhardtii periplasmic CA must be transported across the endoplasmic reticulum in order to be targeted to the apoplast. Our approach to achieve this was to construct a chimeric gene consisting of a CaMV promoter, pCA open reading frame and PinII terminator in the binary plant expression vector pBin19, designated pBin-TL-CA. This construct had a translational fusion between pCA and the signal sequence of the extracellular tobacco peroxidase (Lagrimini et al., 1987) to achieve ER targeting, along with the untranslated leader sequence Ω of tobacco mosaic virus, plus the double 35S promoter for enhanced expression (Gallie and Kado, 1989; Kay et al., 1987). The constructs were transformed into tobacco, and 10 independent transformants were selected for screening. In the initial screening of total

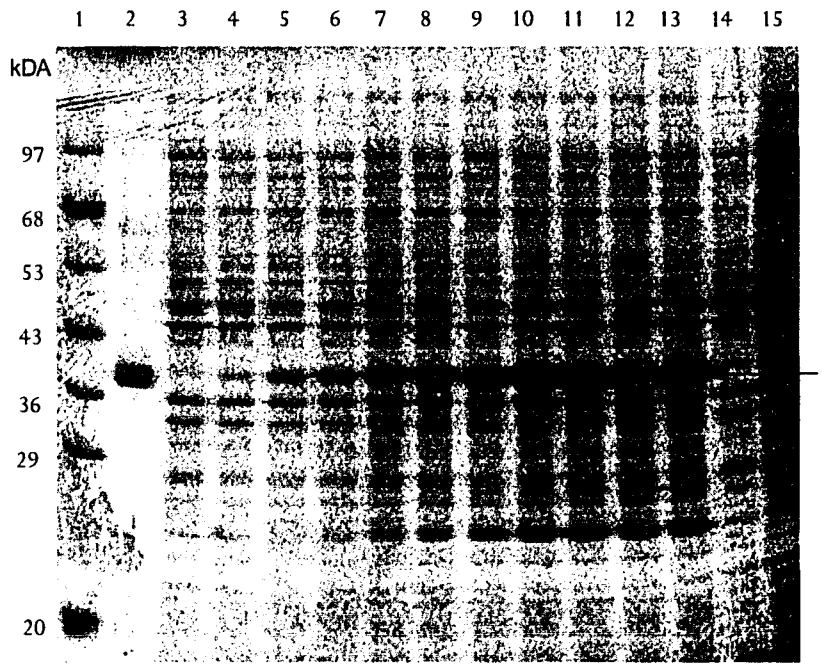

Figure 5. SDS-PAGE showing expression of *C. reinhardtii* periplasmic CA from pET11d-CA in *E. coli*. Lane 1, MW markers; lane 2, purified *C. reinhardtii* periplasmic CA; lane 3, *E. coli* HMS 174 (DE3) with pET11d before IPTG induction; lanes 4 to 13, *E. coli* HMS 174 (DE3) with pET11d-CA at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 hours after the addition of IPTG; lane 14, *E. coli* HMS 174 (DE3) with pET11d 4.5 hours after IPTG induction; lane 15, *E. coli* HMS 174 (DE3) with pET11d-CA after 4.5 hours of growth, with no IPTG added. Arrow indicates 39 kDa peri-CA1 polypeptide.

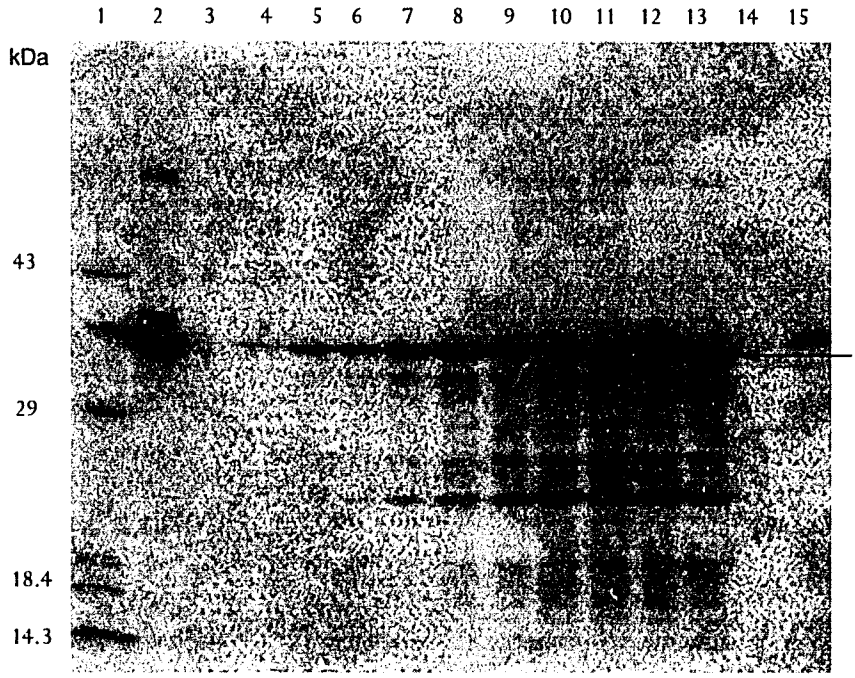

Figure 6. Western blot using affinity purified antiserum generated from *C. reinhardtii* periplasmic CA antigen, showing expression of periplasmic CA from pET11d-CA in *E. coli*. Lane 1, MW markers; lane 2, purified *C. reinhardtii* periplasmic CA; lane 3, *E. coli* HMS 174 (DE3) with pET11d before IPTG induction; lanes 4 to 13, *E. coli* HMS 174 (DE3) with pET11d-CA at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 hours after the addition of IPTG; lane 14, *E. coli* HMS 174 (DE3) with pET11d 4.5 hours after IPTG induction; lane 15, *E. coli* HMS 174 (DE3) with pET11d-CA after 4.5 hours of growth, with no IPTG added. Arrow indicates 39 kDa peri-CA1 polypeptide of interest.

protein extracts from these transgenic plants by immunoblotting using affinity purified peri-CA1 antiserum, all were found to be expressing peri-CA1 (Figure 7). From this group. TL_1, a plant showing a high level of expression (Figure 7) was chosen for further analysis. Northern blot analysis (Figure 8) showed that TL_1, but not untransformed tobacco, contained hybridizable pCA transcripts of the expected size (1.4 kb).

Post-translational processing of periplasmic CA

A total soluble protein extract from TL_1 was subjected to p-aminoethylbenzene-sulfonamide-agarose affinity chromatography (Yang et al., 1985), to purify peri-CA1 present in the extract . This protein, along with purified peri-CA1 from *C. reinhardtii*, was separated on SDS-PAGE. After electrophoresis, proteins on the gel were fixed in a solution containing glutaraldehyde, then Coomassie stained (Figure 9). The *C. reinhardtii* peri-CA1 subunits are of the anticipated sizes (Figure 9, panel A, lane 3), with one broad band (large subunit) around 35 to 37 kDa and a small band (small subunit) migrating at 4 kDa (Kamo et al., 1990). All of the subunits in TL_1 peri-CA1 were of slightly smaller size than occurs in the native algal protein. There were two bands migrating at ca. 34 and 36 kDa and a small band at ca. 3.5 to 3.8 kDa (Figure 9, panel A, lane 4). An immunoblot of purified CA from TL_1 and *C. reinhardtii*, using affinity purified antiserum (see materials and method) detected cross reaction with the large polypeptides but not the small ones (Figure 9, panel B, lanes 3 and 4).

Thymol and sulfuric acid staining to detect glycoproteins confirmed that the large polypeptides were glycosylated (Figure 10). Counterstaining the gel with Coomassie brilliant blue confirmed that the bands detected as glycoproteins, were polypeptides in same positions as anticipated (Figure 10) for peri-CA1.

Localization of periplasmic CA in transgenic tobacco

To determine the location of the peri-CA1 in the plant, leaf tissue was subjected to vacuum infiltration and centrifugation, yielding intercellular fluid (Rathmell and Sequeira, 1974). Soluble proteins were also extracted from the leaf tissue remaining after infiltration/centrifugation yielding a crude residual cellular (intracellular) protein fraction.

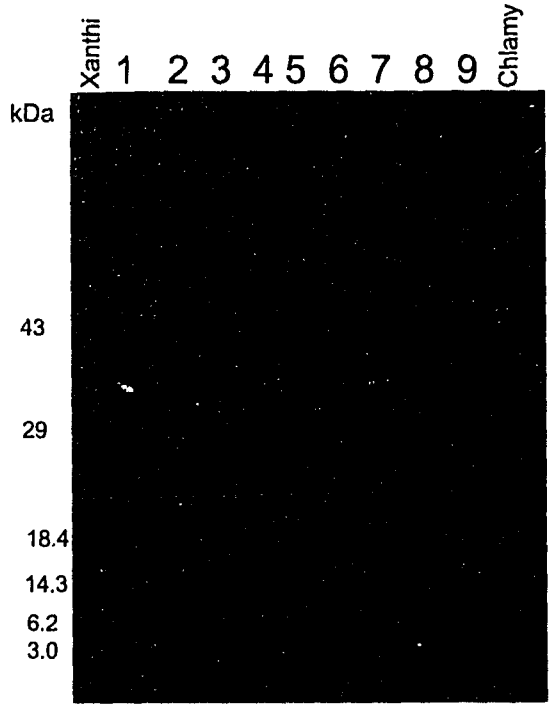

Figure 7. Immunodetection of *C. reinhardtii* periplasmic CA expressed in transgenic tobacco plants. Untransformed tobacco, Xanthi (lane 1), transformed tobacco plants TL_1 to TL_9 (lanes 2 to 10), purified periplasmic CA from *C. reinhardtii* (lane 11).

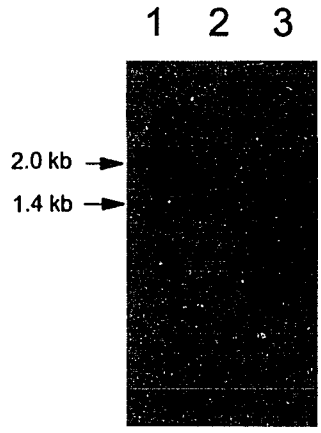

Figure 8. Northern analysis of total RNA from TL_1 transgenic tobacco plant. Total RNA from air induced *C. reinhardtii* (lane 1), total RNA from untransformed tobacco plant (lane 2) and total RNA from TL_1 (lane 3)

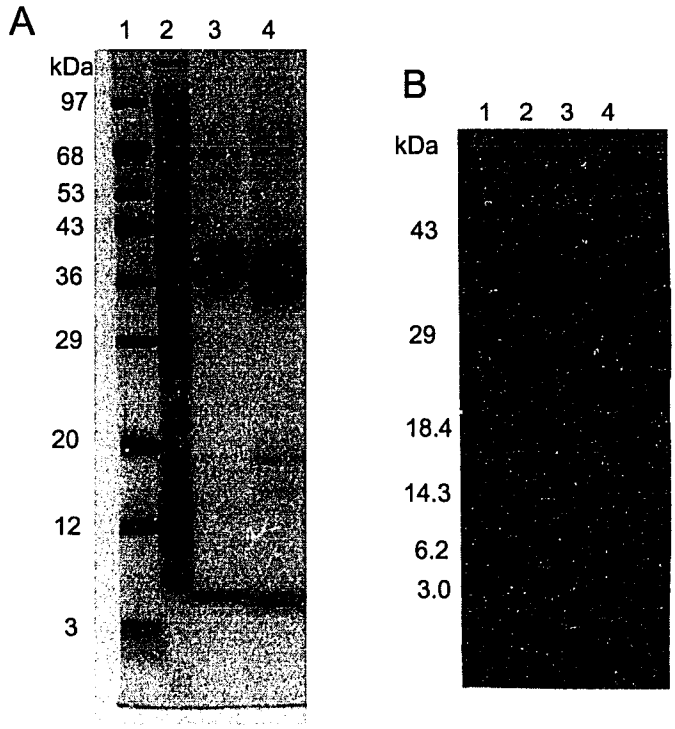

Figure 9. Electrophoretic profiles showing Coomassie brilliant blue staining patterns (panel A) of polypeptides and immunoblots (panel B) of periplasmic CA separated by SDS-PAGE. MW markers (lane 1), total protein from *E. coli* expressing periplasmic CA construct (lane 2), purified *C. reinhardtii* periplasmic CA (lane 3) and purified periplasmic CA expressed in TL_1 transgenic tobacco plant (lane 4).

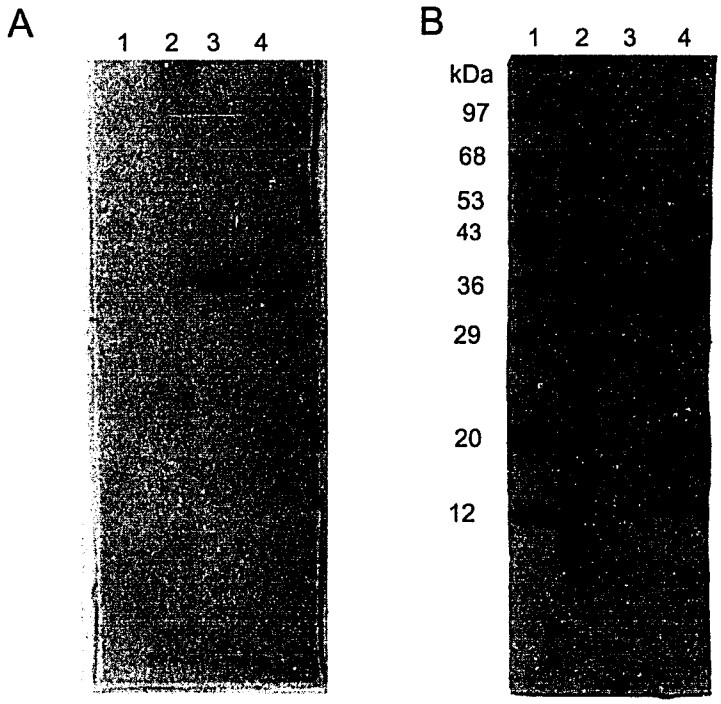

Figure10. Thymol - Sulfuric Acid staining for oligosaccharides attached to proteins (panel A) and Coomassie brilliant blue staining of the same gel (panel B) showing proteins present. MW markers (lane 1), total protein from *E. coli* with pET11d-CA induced (lane 2), purified *C. reinhardtii* periplasmic CA (lane 3) and purified periplasmic CA expressed in TL_1 transgenic tobacco plant (lane 4).

Equal percentages of both (inter- and intracellular) fractions were analyzed for CA, soluble peroxidase (intercellular fluid marker) and NAD-dependent malic dehydrogenase (cytosolic marker) activity. The intercellular fluid fraction contained 35 % of the soluble peroxidase activity and 47 % of the carbonic anhydrase activity present in the total leaf strips (Table 1). In addition SDS-PAGE revealed that the only detected protein in the IF fractions of TL_1 was peri-CA1 (Figure 11). This presence of peri-CA1 was not due to leakage from within the cell, as NAD-dependent malic dehydrogenase activity present the intercellular fluid was less than 3 % of total, after vacuum infiltration and centrifugation. These results suggest that the periplasmic CA protein is being selectively secreted from the leaf cell and accumulates in the apoplastic space.

Comparison of inhibition of CA from *C. reinhardtii* versus TL_1 transgenic

Peri-CA1 activity in *C. reinhardtii* is, like mammalian CA, very sensitive to inhibition by sulfonamide inhibitors, acetazolamide and ethoxyzolamide (Husic et al., 1989). Plant CA however is much less sensitive to these inhibitors (Jacobson et al., 1975; Kandel et al., 1978). The sensitivity of affinity purified periplasmic CA 1 activity in TL_1 by acetazolamide and ethoxyzolamide, was very similar to that of the extracellular enzyme from *C. reinhardtii*, (Table 2), both of which were far more sensitive to the sulfonamide inhibitors than was the native tobacco CA (Table 2). Further the specific activity of affinity purified peri-CA1 from TL_1 and Chlamydomonas were almost identical, 4.4 and 4.2 U/μg, respectively.

Discussion

The targeting of *C. reinhardtii* peri-CA1 to the intercellular space of tobacco plants, and the characterization of this protein as expressed in transgenic tobacco plants are studied in this work. The N-terminal signal peptide coding region of tobacco anionic peroxidase was fused to the open reading frame of *C. reinhardtii* peri-CA1, less the native signal, in the expectation that the expressed protein would be targeted to the ER. The signal peptide was cleaved, and peri-CA1 transported to the apoplast, probably via the default pathway (Denecke, 1990). The presence of peri-CA1 in transgenic plants was detected using affinity

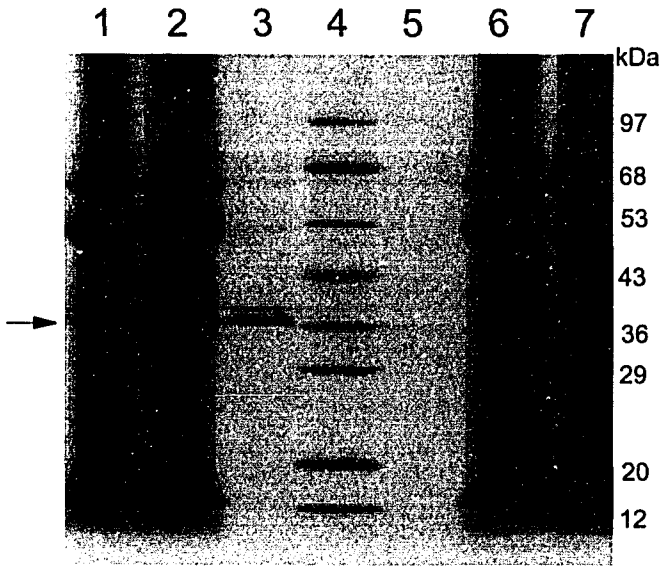

Figure 11. Coomassie stained SDS-PAGE of equal proportions of intercellular, "residual cellular" and total protein from TL_1 and untransformed tobacco plants. TL_1 total (lane 1), TL_1 residual cellular (lane 2), TL_1 intercellular (lane 3), MW markers (lane 4), untransformed intercellular (lane 5), untransformed residual cellular (lane 6) and untransformed total (lane 7). Arrow indicates the position of periplasmic CA in TL_1 lanes, which is absent in untransformed lanes.

Table 1. Percentage of activity of enzymes localized in the intercellular fluid and in the residual cell material, obtained from transformed (TL_1) and untransformed tobacco leaves.

Leaf Strips	Soluble Peroxidase (mU)[1]	Malic dehydrogenase (μmol NADH/min)[2]	Carbonic anhydrase (Units)[3]
Amount present/g fresh weight			
Transformed (TL_1)	33.3	84.53	132.0
Untransformed	37.3	79.20	1.6
Specific activity (/mg protein)			
Transformed (TL_1)	3.8	0.32	50.8
Untransformed	4.4	0.30	*
Intercellular fluid			
% of amount			
Transformed TL_1)	35.3	1.64	47.1
Untransformed	38.9	2.41	*
Residual cell material			
% of amount			
Transformed (TL_1)	64.7	98.36	53.0
Untransformed	61.1	97.59	*

[1] mU, milliunits; amount of enzyme causing an increase of absorbance of 1.0/ min at OD_{470}

[2] μmol NADH/min, μmoles NADH oxidized per minute at 25 °C as measured by decrease in absorbance at 340 nm

[3] Units, (T_b/T_e)-1; where T_b and T_e represent times (blank and sample respectively) at 4 °C for a pH change from 8.3 to 7.3

* No significant carbonic anhydrase activity was detected as assay was for periplasmic CA (measured in the absence of dithiothreitol) not native plant CA, which needs dithiothreitol for activity

Table 2. I_{50} values (M) for sulfonamide inhibition of CA activity in TL_1 transgenic tobacco, *C. reinhardtii* and untransformed tobacco plant by acetozolamide (AZA) and ethoxyzolamide (EZA). Assays for periplasmic CA was done absence of dithiothreitol (DTT), while Xanthi CA was measured in presence of DTT

Sample *	Specific activity	AZA I_{50} (M)	EZA I_{50} (M)
C. reinhardtii periplasmic CA	4.2 units/μg	1.6×10^{-8}	7.4×10^{-9}
TL_1 periplasmic CA	4.4 units/μg	2.0×10^{-9}	2.3×10^{-9}
Xanthi CA	(not available)	1.0×10^{-5}	1.3×10^{-6}

* *C. reinhardtii* and TL_1 periplasmic CA protein extracts were affinity purified. Xanthi CA was measured from crude protein extract (see methods).

purified peri-CA1 antiserum. The expressed CA was active, and in many ways quite similar to the native enzyme in *C. reinhardtii*. This would suggest that the post-translational processing as occurs in the algae is largely conserved in the higher plant.

Several plants were assessed for levels of peri-CA1 by immunoblotting, and varying levels of expression were observed. No peri-CA1 was detected in untransformed tobacco. TL_1 and TL_2 showed the highest level of expression, while TL_5 displayed the lowest (Figure 7). This variability may be a reflection of position effect of insertion of the gene into the plant genome. Nevertheless, a single plant, TL_1, with a high level of expression was chosen for further analysis.

Using northern analysis, detectable transcript levels for pCA of the expected size were found in TL_1, but none could be detected in untransformed tobacco. Since equal amounts of total RNA was used for all samples, the high accumulation of peri-CA1 message in TL_1 is a reflection of the high steady-state level of hybridizable pCA mRNA present in the plant.

Periplasmic CA was purified from TL_1 and *C. reinhardtii*, then subjected to gel electrophoresis and immunoblot analysis, using purified anti-peri-CA1 (Figure 8). SDS-PAGE revealed that the enzyme was processed in tobacco plants fairly similarly as in the algae. There were two large polypeptides migrating around 35 to 38 kDa and smaller polypeptides at around 4 kDa, as expected. The higher molecular weight bands in the plant were smaller (34 to 36 kDa) than those in *C. reinhardtii* (35 to 37 kDa). Also, the small subunit in TL_1 occurred as a "doublet", which was of slightly lower in apparent MW than the single band occurring in *C. reinhardtii*. The differences in size of the large subunits, between the plant and algae could reflect differences in proteolytic enzymes present in the two organisms. The N-terminus of the large subunit may be different in the plant versus *Chlamydomonas*, as the site of cleavage in TL_1, between the tobacco anionic peroxidase signal peptide and peri-CA1 open reading frame may not be exactly the same. The C-terminus of the large subunits between the plant and algae may also different, for similar reasons. Comparison of N-terminal and C-terminal sequences of the two large subunits in both organisms would clarify this. It should however be noted that the differences between the large subunits of TL_1 and the algae could also result from differences in glycosylation patterns in tobacco versus *C. reinhardtii*.

Proteases with slightly different proteolytic sites, at the C-terminal or N-terminal of the small subunit, may be responsible for the small subunit doublet in TL_1 and for the apparent smaller size of the small subunits. Additionally, a few other differences were

discerned between purified periplasmic CA in TL_1 and the algae. Faint bands occurring at 20 kDa and 14 kDa were detected in purified preparations from TL_1, which were absent from *C. reinhardtii*. Whether these could be explained as differences in proteolytic cleavage, or degradation products during enzyme purification is not clear.

Peri-CA1 is synthesized as a precursor of 41.6 kDa, which is processed post-translationally, resulting in a heterotetramer holoenzyme held together with disulfide bonds (Fukuzawa et al., 1990; Kamo et al., 1990; Ishida et al., 1993). It has been reported that castor bean ricin (Butterworth and Lord, 1983; Halling et al., 1985) and legumin of legume seeds (Lycett et al., 1984) are synthesized as precursors, then cleaved into two subunits that remain linked by disulfide bonds. These however are not secreted proteins, but vacuolar proteins, and proteolytic cleavage is thought to occur in the vacuole (Chrispeels and Tague, 1991). In the yeast, *Saccharomyces cerevisiae*, α-factor, a secreted mating pheromone, is excised from precursor polypeptide by the sequential activity of proteolytic enzymes: the KEX2 gene product first cleaves on the carboxyl side of pairs of basic residues; then the STE13 (aminopeptidase) and KEX1 (carboxypeptidase) gene products excise, respectively, Glu-Ala and Asp-Ala pairs and residual lysine residues, generating the mature protein (Bourbonnais et al., 1988). Studies have shown that KEX2 protease is a resident Golgi processing enzyme (Redding et al., 1991). Precisely where in the plant secretory pathway and by what means proteolytic processing of periplasmic CA occurred cannot be discerned from current data. Similarly, details of such processing in *Chlamydomonas* is not known at present. Nonetheless, such processing did occur successfully, resulting in the generation of an active secreted multimeric protein. Further this purified protein separates on SDS-PAGE in a very comparable electrophoretic pattern to the algal protein.

Immunoblot analysis, revealed cross-reaction to the large subunits (34 to 37 kDa) in both tobacco and *C. reinhardtii*, but not the small subunits. This was somewhat surprising, since the antigen used in preparing the antibody was the entire coding region of the pCA gene, less the signal sequence, as expressed in *E. coli*. Special steps may need to be taken in detecting the small subunit because of its size, to ensure that it does not pass through the nitrocellulose membrane during western blot analysis. Multiple membranes and reduced transfer time were attempted, in the hope that the peptide would be retained. It is not clear whether small subunits were not retained on the membrane or were not recognized by the antibodies. The small subunit only makes up 10 % of the total protein mass and was only detected (Coomassie staining), when large amounts of purified protein

were used. At these levels, the 14 and 20 kDa bands in TL_1 were also detected. Such results may imply that a minimum threshold level of this polypeptide is necessary for immunodetection. Nonetheless, immunodetection did confirm that the large subunits of periplasmic CA were present in TL_1.

Analysis (SDS-PAGE and CA activity) of vacuum infiltrate versus total soluble protein of TL_1 show that peri-CA1 accumulates in the apoplastic space of the plant. This suggests that the tobacco anionic peroxidase leader sequence did target the polypeptide to the ER and that the signal sequence was successfully cleaved. Additionally, accumulation in the apoplastic space leads one to conclude that the plant cell successfully secreted the enzyme. It is generally accepted that in yeasts and mammals, specific sorting signals exist which are recognized in the Golgi, that target proteins to vacuoles, lysosomes or re-entry to the ER (Munro and Pelham, 1987; Pelham et al., 1988). Removal of such signals result in "default" secretion of protein. There is also regulated secretion of proteins where release is mediated by extracellular signals (Burgess and Kelly, 1987). It is not clear whether final secretion from the plant cell is via a default pathway or if specific secretory signals exists on the proteins. Nonetheless, the fact that the active holoenzyme accumulates in the apoplast suggest that the post-translational processing between the two organisms is mutually recognizable. Further, since the mature enzyme was comprised of different subunits, of similar sizes as occur in *C. reinhardtii*, would imply that post-translational proteolytic processing was largely conserved.

Sulfonamide inhibition of activity was very similar comparing periplasmic CA from the plant and algae. Native tobacco CA is not as sensitive to sulfonamides (Table 2), as peri-CA1 from TL_1 and *Chlamydomonas* were. Further, native tobacco plant CA needs to be extracted and assayed with the inclusion of a sulphydryl reagent (see materials and methods) to protect the enzyme and remain active (Reed and Graham, 1981), the presence of such a reagent drastically inhibits peri-CA1 activity (Husic et al., 1991). Due therefore to the mutual exclusive nature of the two assays, one can conclude that peri-CA1 is indeed present in TL_1. One can thus assume that because of this similarity in sensitivity of periplasmic CA between the transgenic plant and alga, that the access tunnel to the active sites must be similar, since these inhibitors function by associating with the zinc atom at the active site (Reed and Graham, 1981). Additionally, the specific activity of the purified peri-CA1 from TL_1 and Chlamydomonas were almost identical (Table 2). It is therefore possible that the tertiary and quarternary structures of the enzyme from the algae and

transgenic plant are very similar, again supporting the suggestion of conservation of post-translational processing between the two organisms.

Oligosaccharides on glycoproteins can be detected by staining with thymol and sulfuric acid. Figure 10 shows that the large subunits are glycosylated in both the plant and *C. reinhardtii*. As stated earlier, the large subunits of periplasmic CA in *Chlamydomonas* stains as a doublet of bands after SDS-PAGE, and this is thought to be possibly due to differential glycosylation. Since the doublet also occurs in tobacco, it is possible that the polypeptide is similarly processed in the plant. Current evidence indicates that N-glycosylation occurs co-translationally in plants (Vitale et al., 1993). The number of glycosylation sites and co-translational folding can influence the efficiency of N-glycosylation by restricting access of oligosaccharyl enzymes to relevant sites (Vitale et al., 1993). Additionally, oligosaccharides are further processed in the ER, and still further modifications can take place in the Golgi. It is possible that incomplete or different glycosylation processing of one or more sites, occurred in the tobacco as compared to *Chlamydomonas*. One is tempted to speculate, based on Figures 9 and 10, that glycosylation is not identical in plants versus the alga, bearing in mind that the differences in migration patterns may due to differences in MW of the polypeptides.

Considering the extensive processing required by *Chlamydomonas* periplasmic CA, it is somewhat surprising that it can be processed in transgenic tobacco plants to yield a functional enzyme. Targeting to the plant's ER was achieved by the use of the signal peptide from anionic peroxidase isozyme of tobacco. This isozyme is a glycoprotein that is localized to the extracellular space and thus requires transport across the ER membrane (Lagrimini et al., 1987). During or after the process of translocation across the ER membrane, nascent polypeptides will acquire secondary, tertiary and in many cases quarternary structures and may be modified by the addition of glycans, cleavage by proteolytic enzymes or other processes (Vitale et al., 1993). The tobacco plant processed the polypeptide, ultimately producing a multimeric, glycosylated enzyme, that was secreted to the apoplast. Further this enzyme was active and displayed functional characteristics similar to the native periplasmic CA in *Chlamydomonas*.

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GENERAL SUMMARY

The expression of the coding region of *C. reinhardtii* periplasmic carbonic anhydrase 1 (peri-CA1) in *E. coli* and transgenic tobacco plants was investigated in this study. For bacterial expression, a T7 RNA polymerase/T7 promoter expression systems was used to make two plasmid constructs. In one construct, the signal leader peptide was removed from the periplasmic CA coding region, while in a second construct, the signal peptide was retained. For expression in the tobacco plant, the periplasmic CA 1 native leader sequence was replaced with that from tobacco anionic peroxidase. The plant expression vector construct also included a duplicated 35S promoter from CaMV and the untranslated leader sequence Ω' of tobacco mosaic virus RNA which serves as a translational enhancer to optimize expression.

A very high level of expression of peri-CA1 in *E. coli*, was obtained with the construct that did not include the target leader sequence. The overexpressed polypeptide represented a very significant percentage of total cellular protein after 4.5 hours of growth. In spite of this high accumulation, no peri-CA1 activity was detected in the lysed bacterial cells. The expressed protein was insoluble and present in inclusion bodies. When the *C. reinhardtii* peri-CA1 signal sequence was present, no expressed polypeptide could be detected by immunoblotting, in the periplasm or cytoplasm, although, the presence of this construct inhibited growth of the bacteria.

C. reinhardtii peri-CA1 was successfully expressed in tobacco plants. The enzyme was glycosylated, active and secreted to the apoplast. The holoenzyme in the transgenic tobacco was similarly inhibited by sulfonamides as it is in *Chlamydomonas*, thus affinity purification using p-aminoethylbenzene-sulfonamide could be readily carried out. SDS-PAGE of the purified peri-CA1 from tobacco, revealed a banding pattern similar to that obtained with the alga, except that the subunits expressed in the plant were smaller than their comparable bands in *Chlamydomonas*. The small subunit also stained as a doublet in the transgenic plant, whereas only a single band was is present in the native *C. reinhardtii* protein.

Inclusion bodies, dense aggregates of insoluble protein and RNA, are generally formed following high level expression of foreign proteins in *E. coli* (Schein, 1989). Peri-CA1 is extensively processed post-translationally in *Chlamydomonas*, and it is not surprising that the enzyme was not properly processed in the bacteria. Overexpression

systems, nonetheless, invariably allow for adequate production of foreign polypeptides in *E. coli*, and in this regard our objective was met. Even though we were not able to target peri-CA1 to the bacterial periplasm or produce an active protein, we did produce abundant amounts of non-glycosylated "antigen" for peri-CA1 polyclonal antibody.

We were somewhat surprised at finding that transgenic tobacco plants were able to process and produce active peri-CA1, considering the extensive post-translational processing required to result in the mature enzyme. Results of molecular and biochemical comparison of the peri-CA1 as expressed in tobacco versus *Chlamydomonas* showed close similarity, except for a doublet of bands for the small subunit in tobacco, whereas the alga only has a single band. From these results, it is suggested that post-translational processing of *Chlamydomonas* peri-CA1 is largely conserved in a higher plant.

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