Expression of acetyl-CoA carboxylase promoter: GUS fusions in developing Arabidopsis thaliana

Brad E. Rozema

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Recommended Citation
https://lib.dr.iastate.edu/rtd/21495

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Expression of acetyl-CoA carboxylase promoter:GUS fusions in developing

*Arabidopsis thaliana*

by

Brad E. Rozema

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Plant Physiology

Program of Study Committee:
Eve Syrkin Wurtele, Major Professor
James T. Colbert
Basil Nikolau

Iowa State University

Ames, Iowa

2001
This is to certify that the Master's thesis of

Brad Rozema

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
# TABLE OF CONTENTS

## CHAPTER 1. INTRODUCTION
- Overview
- Acetyl-CoA carboxylase reaction
- Structural forms of acetyl-CoA carboxylase
- Plant acetyl-CoA carboxylase forms and their genes
- Metabolic uses of plant malonyl-CoA
- Expression of acetyl-CoA carboxylase in plants

## CHAPTER 2. EXPRESSION PATTERN OF A CYTOSOLIC ACETYL-COA CARBOXYLASE PROMOTER:GUS FUSION IN DEVELOPING ARABIDOPSIS
- Introduction
- Materials and methods
- Results of promoter:GUS transgene analysis
- Discussion

## CHAPTER 3. EXPRESSION OF PLASTIDIC ACETYL-COA CARBOXYLASE CAC1 PROMOTER:GUS FUSIONS IN DEVELOPING ARABIDOPSIS
- Introduction
- Materials and methods
- Results of cac1 promoter:GUS fusion analysis
- Discussion

## REFERENCES
CHAPTER 1
INTRODUCTION

Overview

Acetyl-CoA Carboxylase (ACCase) catalyzes the reaction of acetyl-CoA and bicarbonate in order to form malonyl-CoA, which in turn is used as a precursor of many biologically important chemicals (Choi 1995). The significance of this reaction can be demonstrated by the fact that nearly all organisms contain some form of this enzyme, many even contain multiple genes or isoforms. One of the reasons for the widespread presence of ACCase is that the conversion of acetyl-CoA to malonyl-CoA is the first committed step in the production of fatty acids (Lane 1974, Wakil 1983). The fatty acids may then be utilized for membranes, structural or protective very long chain fatty acids, or as energy storage. Within bacteria, animal, and fungal cells this is the primary use for malonyl-CoA; plants, however, also use the malonyl-CoA to produce a range of secondary products (Conn 1981, Nikolau 1984). The division of the synthesis of fatty acids and secondary products in plants corresponds to the presence of separate pools of malonyl-CoA within the cytosol and the plastid of the plant cell, whose membranes are impermeable to malonyl-CoA. Since different products are made from the two pools of malonyl-CoA it make sense for the plant to regulate them independently. One way that most plants seem to regulate ACCase and the malonyl-CoA produced is by utilizing isozymes, enzymes encoded by separate genes that differ structurally but have similar functions. Since bacteria, animals, and fungi only have a cytosolic pool of malonyl-CoA they only require a cytosolic ACCase, however, plants are
much more complex. Plants contain both a cytosolic and plastidic form of ACCase. The plant's cytosolic isozyme is similar to that found in the animal and fungal kingdoms, and is encoded for by a small gene family. Depending on the plant species, the plastidic isozyme may be similar to the cytosolic form or it may be closer to the ACCase found in bacteria like *E. coli* (Sasaki 1993, Alban 1994, Konishi 1994). This variability suggests a complex genetic evolution of ACCase within the plant kingdom (Sasaki 1995). In addition to the genetic complexity, the transcription and activation of ACCase allows for more levels of regulation, which are only beginning to be understood.

**Acetyl-CoA carboxylase reaction**

ACCase is a member of the biotin containing family of enzymes, a group of enzymes that all require the attachment of a biotin co-factor in order to transfer a carboxyl group between substrates (Knowles 1989). The similarities between ACCase and the other members of this family, such as propionyl-CoA carboxylase, pyruvate carboxylase, 3-methylcrotonyl-CoA carboxylase, oxaloacetate decarboxylase, glutaconyl-CoA carboxylase, and transcarboxylase (Caffrey 1995), suggest a common ancestry (Samols 1988), however only some, including ACCase, are widespread in modern organisms. In this group of enzymes biotin is covalently bound to a lysine side chain to form biocytin. From a kinetic standpoint the reaction proceeds in two major steps: first is biotin carboxylation to produce carboxyl-biocytin, the second is carboxyl transfer from the carboxyl-biocytin to acetyl-CoA forming malonyl-CoA.

\[
\text{biocytin-ACCase} + \text{bicarbonate} = \text{carboxyl-biocytin-ACCase}
\]

\[
\text{carboxyl-biocytin-ACCase} + \text{acetyl-CoA} = \text{malonyl-CoA} + \text{biocytin-ACCase}
\]
In order to catalyze this reaction the ACCase enzyme contains three functional domains: a biotin carboxylase, which forms the biocytin, a biotin-carboxyl carrier domain, and a carboxyl transferase, resulting in malonyl-CoA and the regeneration of biocytin (Voet 1990).

**Structural forms of acetyl-CoA carboxylase**

There exist multiple forms of ACCase in biology, each of which are composed of three domains that confer a portion of the enzyme's activity. Together these regions combine to give the enzyme its activity. The forms of ACCase are generally classified into three structural groups: heteromeric with enzyme functions on individual polypeptides, intermediate in which some of the functions are combined onto a single polypeptide, and homomeric with all enzyme functions contained in a single multifunctional polypeptide (Caffrey 1995, Choi 1995).

**Heteromeric ACCase**

Heteromeric ACCase is so called because the complete active enzyme is composed of distinct dissociable polypeptide components. The biotin carboxylase and biotin-carboxyl carrier activities are located on separate polypeptides while the carboxyl transferase activity is divided between two types of polypeptides (Choi 1995, Caffrey 1995, Ke 1997). The heteromeric ACCase is localized in the plastids in a wide variety of plants but does not appear to exist in some members of the Graminacea, such as maize, and wheat (these have a homomeric ACCase in their plastids (Konishi 1994, Stumpf 1980, Harwood 1988, Egli 1993, Gornicki 1993)). The heteromorphic form of ACCase is also sometimes referred to as "prokaryotic" due to its similarity in sequence and quaternary structure to that of ACCase found in many bacteria, the best described of which may be *E. coli* (Guchhait 1974, Kondo...
1991, Li 1992a, b). The *E. coli* ACCase contains several levels of complexity. The first level is that the complete active enzyme is composed of three dissociable components (biotin carboxylase, biotin carboxyl carrier and carboxyl transferase), the second is that one of these components is composed of two non-identical polypeptides, carboxyl-transferases alpha and beta (Alex 1989, Muramatsu 1989, Kondo 1991, Li 1992a, b). The bacteria's biotin carboxylase and the biotin-carboxyl carrier are composed of two copies of the polypeptides encoded by the *accC* and *accB* genes, respectively. The carboxyl transferase, on the other hand, is a heterodimer of polypeptides from the *accA* and *accD* genes. A further level of complexity is introduced by the fact that while *accB* and *accC* occur on a single operon, *accA* and *accD* occur separately on the bacterial chromosome (Li 1992a,b); but all genes must be coordinately expressed to form a functional enzyme. The ACCase may also be affected by the cellular environment in terms of energy, substrate, and product requirements.

**Intermediate acetyl-CoA carboxylase**

When two of the activity domains of ACCase are located on a single polypeptide it may be designated as an intermediate form. An example of this form is found in the fungi *Streptomyces aeruginosa* (Hunaiti 1982) and the nematode *Tubatrix aceti* (Meyer 1978). In these organisms the biotin carboxylase and biotin-carboxyl carrier are individual domains on a single polypeptide with the carboxyl transferase domain occurring on a separate polypeptide (Meyer 1978, Hunaiti 1982). The components of the intermediate form of ACCase combine as one biotin carboxylase/ biotin-carboxyl carrier unit and one carboxyl transferase unit; this structure does not disassociate as easily as the heteromeric form of ACCase, suggesting that it contains stronger intra-enzyme bonds (Choi 1995).
**Homomeric acetyl-CoA carboxylase**

The homomeric form of ACCase is distinguished by the presence of all three domains on a single multifunctional polypeptide. Based on amino acid sequencing, this combination of functions appears to be of evolutionary origin, possibly evolving from separate functions in other polypeptides to form a fused homomeric ACCase (Knowles 1989). The domains are arranged linearly along a 230-265 kDa polypeptide (Yanai 1995) separated by structural and/or regulatory amino acids. This form of ACCase is most commonly found in the cytosol of eukaryotic organisms: chicken, rat, human (Lopez-Casillas 1988, Takai 1988, Ha 1984), yeast (Walid 1992, Hasslacher 1993), plant (Gorniki 1994, Roesler 1994, Schulte 1994, Shorrosh 1994, Yanai 1995), and algae (Roessler 1993), but also may be localized to the plastids of some Graminacea (Egli 1993, Nikolau 1984, Choi 1995). As with the heteromeric and intermediate forms, the activity of the enzyme maybe influenced by a number of factors. One of these factors is that the active enzyme is usually composed of more than one multifunctional polypeptide, ranging from two in mammals and yeast to eight in some plants (Choi 1995), that must be properly assembled within the cytoplasm. The enzyme regulation between plants and mammals or yeast also differs. In plants both short and long term regulation have been investigated and appear to be at the transcriptional level but are not well understood (Yanai 1995). In mammals short term regulation appears to relate to the cells energy status. When a cell has plenty of energy and can afford to store some reserves, ACCase is dephosphorylated, reducing its susceptibility to inhibition by palmitoyl-CoA, a product of fatty acid biosynthesis; thus more carbon may be directed to fatty acid storage molecules without feedback inhibition. When ACCase is phosphorylated, it again becomes
sensitive to palmitoyl-CoA and is inhibited by its own downstream product (Thampy 1988, Kim 1989, Winz 1994).

**Plant acetyl-CoA carboxylase forms and their genes**

Both the heteromeric and homomeric ACCase are found among higher plants (Sasaki 1995). A homomeric ACCase has been found in the cytosol of every plant studied (Caffrey 1995), with soybean (Anderson 1995, 1996) and *Arabidopsis* (Yanai 1995) containing multiple cytosolic enzymes. The plastidic pool of malonyl-CoA is generated from a separate isoform. Whether this is a heteromeric or homomeric enzyme varies by species (Egli 1993, Choi 1995). The redundancy of the ACCase enzyme in the plastid and cytosol reflects the requirement for malonyl-CoA in both subcellular areas, the impermeability of membranes to malonyl-CoA (Yanai 1995), and allows for separate regulatory pathways.

**Evolutionary origins of plant acetyl-CoA carboxylase**

Cytosolic homomeric ACCase is a member of a large gene family, the members of which probably developed in a primordial eukaryotic cell. This theory is supported by the similarity of enzyme sequences and motifs in the members of the biotin-containing enzyme gene family found throughout the plant, animal, and fungal kingdoms.

The presence of a prokaryotic heteromeric form of ACCase in some higher plants may be explained by the endosymbiotic theory of the origin of plastids (Margulis 1981). The endosymbiotic theory states that plastids were once free living cyanobacteria-like organisms that were engulfed and incorporated into primitive eukaryotic cells. Over time some of the plastid genes migrated to the host cell nucleus, as is the case with *cacl* (chloroplastic acetyl-CoA carboxylase), the heteromeric ACCase biotin-carboxyl carrier gene, while others
remained in the plastid genome, like $accD$, a heteromeric ACCase carboxyl transferase component (Sasaki 1993). In certain higher plants the functional heteromeric ACCase has been lost from the plastid and replaced with a form of the cytosolic homomorphic enzyme (Konishi 1994, Stumpf 1980, Harwood 1988, Egli 1993, Gornicki 1993). One possible mechanism for this process is suggested by the maize genome. The plastidic homomorphic ACCase was apparently generated from a gene duplication of the cytosolic enzyme that over time became targeted to the plastid (Caffrey 1995). Once the nuclear encoded homomorphic form was present in the plastid there may have been less selective pressure to maintain the plastidic heteromeric form; eventually these genes were lost as the homomeric enzyme took over completely for the heteromeric form (Choi 1995). Other plants, such as Arabidopsis and soybean, also have duplicated cytosolic ACCase genes. It is possible these are used to regulate the supply of malonyl-CoA to the cytosol through differential expression, while the plastidic heteromeric enzyme is retained to provide malonyl-CoA for fatty acid synthesis.

**Plastidic acetyl-CoA carboxylase**

Because of the endosymbiotic origin of plastids in plant cells and the interactions of their separate genomes, the ACCase found within the plastid may be either of the eukaryotic (homomeric) or prokaryotic (heteromeric) form in different species with different evolutionary histories. A plastidic homomorphic ACCase has been identified in some graminaceous monocots, such as maize, wheat, and rice (Choi 1995). The maize plastidic ACCase consists of eight identical multifunctional polypeptides (Nikolau 1984) which is similar to the homomeric cytosolic form but vary in their sensitivity to certain herbicides (Ashton 1994). This plastidic isozyme is also much more abundant within the plant then the cytosolic isoform (Egli 1993). The plastidic ACCase found in many dicots, such as
**Arabidopsis** (Choi 1995), spinach (Kanangara 1972, Mohan 1980), tobacco (Shorrosh 1995, 1996), and in some monocots, such as leek (Caffrey 1995), and barley (Kanangara 1975, Reizel 1976), is of the heteromeric form. The heteromeric form is sometimes referred to as prokaryotic due to its similarity to the ACCase found in the bacterium *E. coli*. The *E. coli* ACCase consists of several unifunctional polypeptides acting together to form an active enzyme. In plants the study of proteins and genes has shown that the heteromeric ACCase is composed of four polypeptides, grouped into three components, each with one of the holoenzyme functions of biotin carboxylase, biotin-carboxyl carrier, or carboxyl transferase. The first two polypeptides are responsible for the biotin carboxylase and biotin-carboxyl carrier functions while the third and fourth polypeptides are both associated with the carboxyl transferase activity (Ke 1997). In a few species all of the components have been identified, but how they are actually combined to form the active plastidic enzyme, in terms of numbers of units, arrangement, and bonding, is not clear (Sasaki 1993, Choi 1995, Shorrosh 1995, 1996, Ke 1997, Sun 1997).

The plant plastidic heteromeric ACCase is not only similar to *E. coli* in that it is composed of several unique polypeptides, but also in that the genes encoding the plant polypeptides correspond to the *accA*, *accB*, *accC*, and *accD* *E. coli* genes. One significant difference, however, is the distribution of the plant genes between the plastids circular chromosome and the nuclear genome. *Arabidopsis*, for example, contains four genes encoding the heteromeric components of its plastidic ACCase. The three nuclear genes *cac1*, *cac2*, and *cac3* correspond to the *E. coli* biotin-carboxyl carrier gene *accB*, the biotin carboxylase gene *accC*, and the carboxyl transferase gene *accA* (Choi 1995, Sun 1997), respectively. The fourth *Arabidopsis* gene, encoded in the plastid chromosome, is known as
accD and encodes the second component of the carboxyl transferase as does the identically named *E. coli* accD gene (Ke 1997).

Plants which contain a homomeric plastidic ACCase may lack some or all of the prokaryotic form genes (Choi 1995). Wheat and rice have been demonstrated to lack the accD homolog, while maize lacks both the accD and cac1 homologs (Nagano 1991, Sasaki 1993, Choi 1995). Partial cDNAs have been used to map the maize cytosolic and plastidic homomeric ACCases to separate nuclear chromosomes (Caffrey 1995)

**Cytosolic acetyl-CoA carboxylase**

While the plastidic form of ACCase may vary between species the cytosolic form has been consistently identified as being a homomeric isoform in species as varied as alfalfa (Shorrosh 1994), *Arabidopsis* (Yanai 1995), maize (Egli 1993), pea (Alban 1994), soybean (Caffrey 1995, Anderson 1995, 1996), and wheat (Gornicki 1994). When the cytosolic protein is isolated from plants like maize, pea, soybean, or the algae *Cyclotella cryptica* it is found to consist of multifunctional polypeptide subunits ranging from about 230 to 265 kDa in size (Slabas 1985, Charles 1985, Hellyer 1986, Roesler 1993, Egli 1993, Alban 1994, Ashton 1994, Yanai 1995). The multiple domains of the peptides coincide with the biotin carboxylase, biotin-carboxyl carrier, and carboxyl transferase functions of the enzyme but are not active by themselves outside of the cell. The active enzyme *in vitro* is composed of at least two subunits working together, as demonstrated in parsley, pea, and maize (Egin-Buhler 1983, Alban 1994, Ashton 1994).

As stated previously, a plant may contain not only heteromeric and homomeric isoforms of ACCase, but also multiple cytosolic homomeric isoforms. *Arabidopsis* and soybean each contain two cytosolic isoforms that are known as ACC1 and ACC2 in
Arabidopsis (Yanai 1995) and ACCaseA and ACCaseB in soybean (Anderson 1995, 1996, Caffrey 1995). The two cytosolic enzymes in each species are very closely related at the amino acid level with 90% identity in Arabidopsis (Yanai 1995) and 96% identity in soybean. Between species the four isoforms are also related with 79% or greater identity between all combinations of peptides. Despite their similar function and protein structure, the Arabidopsis isoforms have been demonstrated to be differentially expressed during development and under differing environmental conditions. The two forms may allow the plant to regulate the supply of malonyl-CoA in response to various stimuli (Yanai 1995).

In addition to studies of the deduced amino acid sequence the cytosolic ACCase has been investigated at the genetic level. Several species of plants including alfalfa (Shorrosh 1994), maize (Ashton 1994), wheat (Gornicki 1994), Arabidopsis (Yanai 1995), and soybean (Caffrey 1995) have yielded cDNAs encoding homomeric ACCase. Furthermore the complete gene sequences for ACC1 and ACC2 from Arabidopsis and ACCaseA and ACCaseB from soybean have been determined. The study of these genes has revealed further similarity between the plant homomeric ACCases. All genes are approximately 10 kb in length with mRNA transcripts of about 7500 bp and peptides of 2200 amino acids (Yanai 1995, Anderson 1995, 1996). In addition to the raw sequence the structure of the genes is similar, with introns and exons at equivalent positions, although there are small DNA deletions and additions to individual introns and exons. The physical location of the Arabidopsis genes has been determined to be a tandem duplication within a 25 kb region close to the middle of chromosome 1 (Yanai 1995).
Metabolic uses of plant malonyl-CoA

Plastidic uses of malonyl-CoA

Within the plastids of plants the reaction carried out by either the heteromeric or homomeric ACCase results in a single membrane impermeable product: malonyl-CoA. Once malonyl-CoA is made in the plastids, its principal use is to donate C2 units to the de novo production of fatty acids (Ohlrogge 1979, Stumpf 1987, Harwood 1988). In Arabidopsis the newly produced fatty acids consist mainly of palmitate (C16) and stearate (C18) that may pass through the plastid membranes and into the cytosol. The importance of this process can be demonstrated by the fact that the palmitate and stearate produced are the only de novo source for membrane phospholipids, seed oil triacylglycerides (Ke 1997), and the fatty acids found in waxes (Kolattukundy 1976), without which a cell cannot function. In order to form many of these compounds, however, the original fatty acids must be extended or modified in the cytosol.

Cytosolic uses of malonyl-CoA

In contrast to the malonyl-CoA produced by ACCase in the plastid of plants, or cytosol of animals and fungi, a plant's cytosolic pool of malonyl-CoA is not involved in de novo fatty acid synthesis. A plant cell, instead, uses its cytosolic malonyl-CoA to form very long chain fatty acids, secondary compounds, and malonylated metabolites (Taiz and Zeigler 1991, Yanai 1995). One possible explanation for this ability relates back to the endosymbiotic origin of plastids. Once a plastid had been incorporated in the ancient plant cell the host was able to make use of the fatty acids produced by the symbiont. This, in turn, reduced the demand for cytosolic malonyl-CoA to be incorporated into fatty acids and allowed for the development of alternative uses for the ACCase product.
One of the most widespread uses of malonyl-CoA is the elongation of existing fatty acids. In the cytosol of *Arabidopsis* this means that the C16 and C18 acids exported from the plastid are extended to very long chain (VLC) fatty acids of C20 to C30 (Kolattukudy 1980, 1987, Lessiere 1985). These VLC fatty acids may then be used as energy storage molecules in some seed oils (Pollard 1980) or may be modified and incorporated as waxes into the plant cuticle or suberin (Taiz and Ziegler 1991). The cuticle and suberin both act as barriers for the cell to prevent water loss and pathogen entry. The cuticle is produced as droplets of wax in epidermal cells that are then secreted to create a surface coating over the exterior of the plant. Suberin not only acts as a sealant for the exterior of the plant in bark and the outer walls of roots, but also functions to seal wounds from disease, physical damage, or abscission (Rittinger 1986, Taiz and Ziegler 1991). It may also function as an interior barrier in the Casparian strip of the root endodermis.

The term "secondary compounds" in plants describes a class of metabolites which are not directly required for the essential life functions of the plant but are present to enhance a plant's survival (Taiz and Zeigler 1991). The wide variety of secondary chemicals which require malonyl-CoA may be categorized into several structural classes: flavonoids and related compounds (Stafford 1995), stilbenes and related compounds (Gross 1981), acridones (Junghanns 1995), and quinones (Leistner 1981). Flavonoids include a range of chemicals with similar biosynthetic pathways and phenolic containing structures. The flavonoids are divided among anthocyanin pigments, flavones, and flavonols that absorb ultra-violet light energy (Robberecht 1978), and defensive isoflavonoids (Taiz and Ziegler 1991). The stilbenes are synthesized via the shikimate pathway and act as inducible phytoalexins (Gross 1981, Brignolais 1985, Borejsza-Wysocki 1996, Beckert 1997). Another way in which plants
protect themselves is through the production of mutagenic, anti-viral, or anti-herbivory alkaloids like the acridones (Junghanns 1995). The final group of secondary metabolites derived from malonyl-CoA is the quinones and their derivatives (Packter 1980, Leistner 1981) which act to limit herbivory.

The third class of malonyl-CoA derived compounds consists of malonylated metabolites. In this group the malonyl portion of malonyl-CoA is attached to a second compound to influence its storage and use within the cell. If a toxic xenobiotic compound is introduced into a cell it may be converted to a more water-soluble form by the addition of malonyl, in order that it may be stored in the vacuole away from vital cell components (Sandermann 1991, Taiz and Ziegler 1991). Malonylation also affects natural cell compounds by changing their reactivity. For example, malonyl may bind irreversibly to the ethylene precursor, 1-amino-1-cyclopropane carboxylic acid, preventing the formation of that powerful hormone (Gallardo 1991, Kionka 1984), or it may bind to D-amino acids preventing their incorporation into proteins which are normally composed of only L-amino acids.

**Expression of acetyl-CoA carboxylase in plants**

Due to the importance of the ACCase reaction and to the complexity of the enzyme system, the localization and expression of the ACCase genes has been the subject of several studies. The plastidic isozyme is detected at its highest levels in tissues that are rapidly growing or expanding and have high fatty acid requirements. For example, the *Arabidopsis cac1* gene is expressed throughout young, growing leaf epidermis and mesophyll cells but is present at much lower levels in older, expanded leaves (Ke 2000). *Arabidopsis seeds*
accumulate oil for energy storage as they mature, meaning the fatty acids required must be produced by plastidic ACCase genes that encode for the plastidic ACCase. These genes show a coordinated expression pattern, increasing and decreasing in conjunction with lipid deposition (Ke 2000). The cytosolic ACCase also adjusts to the cell's demand for malonyl-CoA in response to developmental and environmental signals. Evidence of the differential expression of cytosolic ACCase has been observed in *Arabidopsis*. As described previously, *Arabidopsis* contains two cytosolic ACCase genes, *ACC1* and *ACC2*, which are driven by different promoters. The *ACC1* mRNA is detected in developing leaves, seedlings, and siliques while *ACC2* is expressed at lower levels in leaves and seedlings but appears to be absent from siliques (Ke 2000). The presence of the *ACC2* transcript at lower levels may allow for its activation in response to an environmental signal, like wounding, which would require additional malonyl-CoA products, while *ACC1* may provide for the cells day-to-day requirements.
CHAPTER 2

EXPRESSION PATTERN OF A CYTOSOLIC ACETYL-COA CARBOXYLASE PROMOTER:GUS FUSION IN DEVELOPING ARABIDOPSIS

Introduction

In plants the cytosolic ACCase catalyzes the reaction of acetyl-CoA and bicarbonate to form a pool of malonyl-CoA. Unlike plastid-lacking organisms, this malonyl-CoA is not used for de novo fatty acid synthesis but instead serves a variety of secondary purposes. Within the cytosol of Arabidopsis this malonyl-CoA is used to extend fatty acids exported from the plastids for storage oils and waxes, as well as forming a number of secondary compounds to cope with biotic and abiotic stresses (Conn 1981). The cytosolic ACCase provides a model system for study; not only is it involved in the housekeeping and storage functions of the cell but also in the response to stresses. Furthermore it consists of a number of components and control points, which may be investigated to expand our understanding of the role of this enzyme in the larger picture of plant metabolism.

The homomeric cytosolic ACCase is composed of two identical 250 kDa subunits (Egin-Buhler 1980, Egli 1993, Gornicki 1994, Alban 1994, Roesler 1994, Shulte 1994, Konish and Sasaki 1994, Sasaki 1995). In Arabidopsis these proteins are coded for by two genes, ACC1 and ACC2 that appear to be the result of a gene duplication event near the center of chromosome 1. To better study these genes, they have been cloned and sequenced along with the surrounding DNA (Ke 2001) that may include the promoter sequences. Initial analysis of both genes' expression was carried out by northern blotting and in situ hybridizations using radiolabelled RNA probes (Ke 2000, 2001). Each of these techniques
contains inherent limitations: northern blots allow for general analysis of mRNA quantity but not fine localization, while in situ hybridizations are limited to microscopic sections from an entire plant. In the case of ACC1 and ACC2 these techniques were further complicated by the great similarity between the two genes, which allows for the probability of cross-hybridization of probes during the reactions.

In order to clarify and extend the previous results an analysis of promoter:GUS fusions was undertaken. By utilizing only the upstream regions of the genes, which vary significantly, the problem of gene similarity could be overcome. Additionally, the GUS reporter gene allows for expression analysis on a larger scale than in situ hybridizations, such as entire seedlings, but also retains a measure of detail that can be resolved with microscopic techniques. In addition to the study of cytosolic ACCase during seedling and fruit development, a number of environmental stresses were investigated to test the gene's response. Since malonyl-CoA is used in the production of flavonoids and anthocyanins, environmental perturbations including blue light stress, cold, and etiolation, which influence synthesis of stress compounds (Kubasek 1992, Noh 1998, McKown 1996), were utilized.

**Materials and methods**

**Transformation and selection of plant material**

*Arabidopsis* plants containing an ACC2 promoter:GUS transgene were obtained from Dr. Basil Nikolau (Iowa State University) at the T1 generation. Transformation was carried out via dipping flowering plants in a liquid culture of *Agrobacterium tumefaciens* (Clough and Bent 1998) containing the pBI101.2 vector plasmid (Clonetech). The plasmid, in turn, contained a 2.8 kb ACC2 promoter fragment fused to the β-glucuronidase reporter gene,
termed ACCII, and a kanamycin resistance marker. After initial selection of the T₀ plants on kanamycin, the resistant lines became contaminated with fungi and mildew during storage. In order to recover the transformed lines and ensure they were homozygous a further growth and selection procedure was necessary. The four initial samples of ACC2:GUS were grown out on soil and the seeds resulting from individual plants were tested for kanamycin resistance. Of the 182 ACCII T₂ plants screened four ACCII lines showed some antibiotic resistance and were tested at the T₃ generation for transgene homozygosity. Ultimately two lines, which showed near complete antibiotic resistance and consistent reporter gene expression, were chosen for study.

**Growth conditions of plant material**

*Arabidopsis thaliana*, ecotype Columbia, seeds were surface sterilized for 7-10 minutes in an aqueous solution of 50% bleach (2.5% hyperchlorite) and 0.02% Triton X-100 then rinsed three times with sterile distilled water. The seeds were placed in petri plates containing 4.3 g/L MS salts (Sigma), 1X B5 vitamins (Sigma), 10 g/L sucrose, 0.5 g/L MES, 0.8% agar at pH 6.7, (for selection 50 µg/ml kanamycin was added) and parafilm sealed after drying. The plates were placed under constant illumination at 28°C for up to 14 days (unless otherwise specified) before the plants were transferred one-by-one to 4 inch pots containing Sunshine LC mix 1 (SunGro Horticulture). They were then allowed to mature, with similar light and temperature conditions. After the initiation of flowering individual flowers were tagged using colored thread on the first day after flowering (DAF) so that they might be harvested at specified times during flower and silique development defined by Bowman (1994).
Stress inducing growth conditions

For the purpose of testing the response of ACC2 to stress inducing conditions, plates of seeds were prepared as describe above but placed into different growing conditions, plants were then collected for GUS staining at several time points. A cold temperature stress was simulated by the use of a cold room to maintain a temperature of 10°C with light provided by fluorescent bulbs at a similar intensity as the standard conditions. Plants were also grown under blue light fluorescent tubes, Phillips F40 BL (350-400nm) at 28°C and 65µE to induce UV stress and anthocyanin production (Kubasek 1992, Noh 1998). As a test for the light requirement of ACC2 expression some plates were placed in a dark room with no or only faint green light exposure before collection. Control samples were grown under standard light and temperature conditions and processed at the same time as the experimental groups to limit variations.

Histochemical analysis of GUS activity

GUS expression was analyzed in two lines of the ACCII transgenic plants, with similar results for both lines. Plant material was placed whole (seedlings and flowers) or partially dissected (siliques) into a staining solution of 30 mM NaH2PO4, 50 mM Na2HPO4, 8 mM EDTA, 0.12% Triton X-100, 4 mM K3 [Fe (CN) 6], 4 mM K4 [Fe (CN) 6] · 3H2O, 20% methanol, and 1.53 mM X-Gluc (5-bromo-4-chloro-3-indoyl- (D-glucuronic acid)) (Jefferson 1987) and vacuum infiltrated for 10 minutes at -20 in. Hg. Samples were incubated at 37°C for 3 hours (germinating seed) to 18 hours (all other samples) to allow for GUS product accumulation. Chlorophyll was cleared from stained tissue by immersion in 70% ethanol, which was also used to store the samples. Fully processed samples were photographed using a dissecting stereomicroscope and Kodak Elite Chrome 160T film. The resulting
photographic slides were then computer scanned and processed using Adobe PhotoShop 5.0.2® for PowerMacs. All experiments were carried out at least twice with 10-20 samples collected and processed for each transgenic line per repetition.

Results of promoter:GUS transgene analysis

To expand our knowledge of the regulation and expression of the cytosolic homomeric ACCase in developing *Arabidopsis* a promoter:GUS fusion of *ACC2* was inserted into the genome and its expression analyzed. The conditions and tissues used during these experiments allowed for the study of the requirement of cytosolic ACCase in developing flowers and seed containing siliques as well as germinating seeds and young plants under various stresses.

Expression of promoter:GUS transgenes in flowers and siliques

Cytosolic ACCase is thought to be involved in the extension of fatty acids, for storage oils, membranes, and waxes, as well as the production of many secondary compounds. The expression patterns observed during silique development support these roles. *ACC2*:GUS expression is detected in several different tissues as the flowers and siliques develop. *ACC2*:GUS expression is initially localized to the very young ovules (fig. 1-E). At a later stage of flower development *ACC2*:GUS expression is restricted to the anthers, in which the staining becomes concentrated to the pollen grains themselves, through dehiscence (figs. 1-E, 1-F). After fertilization and into the early stages of seed development, low levels of GUS staining are visible in the seeds, although this is not always consistent, possibly due to difficulties with the X-Gluc solution's penetration into the seed (figs 1-G to 1-J).
Figure 1. Localization of ACC2:GUS staining in developing Arabidopsis.

Transgenic Arabidopsis containing an ACC2 promoter:GUS fusion were stained with X-GLUC solution for 3 hours (A) or 18 hours (B-J), before clearing with ethanol and photography. (A) seedling 2 days after planting (DAP); (B) seedling 5 DAP; (C) seedling 14 DAP; (D) leaf of seedling 18 DAP; (E) stage 8 flower bud (Bowman 1994); (F) stage 11 flower bud; (G) stage 13 flower; (H) flower 1 day after flowering (DAF); (I) silique 5 DAF; (J) silique 7 DAF. Bar = 1 mm in (A), and (F-H); 500 µm in (E); 5 mm in (B-D), (I), and (J).
**Seedling expression of promoter:GUS transgenes**

Expression of the \textit{ACC2}:GUS transgene was tested in seeds two days after imbibition, with the resulting stain concentrated in the cotyledons and root hairs (fig. 1-A). In seedlings five days after plating \textit{ACC2}:GUS expression was highest in the shoot apex and vasculature of the cotyledons, slightly reduced levels are present in the rest of the cotyledon tissue. In roots, expression is at a lower level and is concentrated to the root tip and vascular cylinder (fig. 1-B). As the seedling matures the level of staining diminishes in the shoot, while expression in the root becomes more intense in the same tissues as found in younger plants (fig. 1-C). Leaf expansion continues as \textit{ACC2}:GUS expression declines, becoming localized to the trichomes before falling below detectable levels (fig. 1-D).

**Environmental stresses and seedling expression of promoter:GUS transgenes**

Cytosolic malonyl-CoA is required for the production of many secondary metabolites active in plant protection. In order to test if there is an alteration of \textit{ACC2} expression in response to stresses, seedlings were grown under differing regimes. Low temperature and blue light were chosen for their ability to induce anthocyanin and flavonoid production in other plant studies (Kubasek 1992, Noh 1998, McKown 1996). Flavonoids and anthocyanin require malonyl-CoA for their synthesis. Constant dark was also tested for effects on the expression of cytosolic ACCase, while growth under constant light at 28°C established a standard for comparisons.

The patterns of GUS expression obtained under the stress conditions were similar to that previously described for seedling grown under standard conditions, with the added dimension of alterations in growth patterns. Plants germinated in the dark showed classic
etiolation growth patterns, including extended hypocotyls and delayed cotyledon expansion. Under these conditions, visible $ACC2$:GUS staining of the shoot was limited to the shoot tip with a small amount also in the root hairs (fig. 2-D and 2-E). After seven days dark-germinated seedlings were transferred to constant light, and at the end of another seven days the plants were analyzed for GUS expression. The initially etiolated plants now showed several expanding leaves with stain, as well as GUS product accumulation in the apical meristem and root tip regions (fig. 2-F). For comparison, plants germinated for seven days in constant light were transferred to the dark for a week before GUS staining. These plants had growth and staining patterns of light-grown seedlings (fig. 2-B). But when transferred to the dark displayed extended hypocotyls and elongated pedicles on newly developed leaves. The $ACC2$:GUS expression pattern in these plants was adapted to this mixture of growth patterns. The shoot tip retained the highest level of expression, young unexpanded leaves on extended pedicles stained throughout the blade tissue, older leaves showed reduced expression, and root tips continued to be blue (fig. 2-G). $Arabidopsis$ plants germinated under standard conditions for five days then grown in a lit cold room displayed growth and staining patterns similar to those grown at 28°C but were delayed in their development. Cold-grown plants at seven days after plating showed significant staining in the shoot tip, cotyledons, and root hairs, but had not developed any true leaves (fig. 2-H). By fourteen days after plating the cold stressed plants showed a reduction in staining of the now expanded cotyledons and small true leaves; at the same time the shoot tip, root hairs and lateral meristems expressed the $ACC2$:GUS construct similarly to less developed plants grown under standard conditions (fig. 2-I compare to 2-A). Finally, growth under a blue light was tested to determine the
Figure 2. Stressed seedling expression of ACC2:GUS transgene.

*Arabidopsis* seedlings were subjected to dark, cold and UV light stresses (described in text) to test the response of the ACC2 promoter:GUS fusion. At specified times after planting and stress treatment, whole seedlings were stained for GUS activity and ethanol cleared before being photographed. Light grown seedlings: (A) 5 DAP; (B) 7 DAP; (C) 14 DAP. Dark stressed seedlings: (D) 5 DAP; (E) 7 DAP; (F) dark for 7 DAP then light to 14 DAP; (G) light for 7 DAP then dark to 14 DAP. Cold stressed seedlings: (H) 7 DAP; (I) 14 DAP. Blue light stressed seedlings: (J) 7 DAP; (K) 14 DAP. Bar=2 mm in (A); 5 mm in (B), (D), (F), and (H-K); 1 mm in (C), (E), and (G),
cytosolic ACCase’s response to photo-oxidative stress. At seven days after plating the seedlings showed less ACC2:GUS expression than the white light grown plants, with a more mottled staining pattern in the cotyledons (fig. 2-J). At fourteen days after plating, staining was limited to the shoot tip and small areas of the root (fig. 2-K). After the final collection for GUS analysis the remaining blue light-grown plants were observed to have some white areas on the leaves and cotyledons that may have been due to photo-bleaching and these may have contributed to the observed reduction in growth and staining (not shown).

Discussion

*Arabidopsis* contains two forms of ACCase, a plastidic heteromeric and a cytosolic homomeric isozyme. The plastidic isoform produces malonyl-CoA used for the production of *de novo* fatty acids, while the homomeric’s malonyl-CoA is utilized in the elongation of fatty acids and the synthesis of secondary compounds. The differential control between the housekeeping and specialized functions of ACCase may be due to multifunctional promoter and enhancer elements influencing the ACCase’s genes expression. In this study we begin an analysis of ACC2 promoter driven expression during development. The *Arabidopsis* cytosolic pool of malonyl-CoA is produced by two homomeric ACCases, *ACC1* and *ACC2* (Yanai 1995). These tandem genes appear to be the product of the type of local gene duplication event common in *Arabidopsis* (Lin 1999, Blanc 2000). When the original cytosolic ACCase gene was duplicated it expanded the biotin-containing enzyme gene family to contain another member. Walsh (1995) speculated the presence of a new gene allows for its adaptation to fulfill a specific role in the plant cell by the alteration of its structure of expression relative to the starting material. To study the potential significance of these
alterations in the ACCase duplication event an ACC2 promoter:GUS fusion was transformed into Arabidopsis and analyzed at various points in development and under different environmental conditions.

The results of this analysis demonstrated a complex, highly regulated expression pattern for the cytosolic ACCase. Accumulation of cytosolic malonyl-CoA derived products (which do not include de novo fatty acids) may be used to explain most of the staining patterns observed. One example of this phenomenon is the frequently observed expression of cytosolic ACC2:GUS in young expanding organs, such as cotyledons, leaves, petals, sepals, and root tips. These tissues require malonyl-CoA for the synthesis of: elongated fatty acids in cuticles, flavonoids, or other secondary compounds. Another occasion which requires malonyl-CoA is the synthesis of elongated fatty acids in tapetal cells shortly before the completion of pollen development (Wu 1999), this is also visualized in our experiments (fig. 2-F). In addition to the patterns of expression which can be readily explained, the use of the promoter:GUS fusion revealed some less expected results. The localization of ACC2:GUS product in trichomes, root hairs and vasculature, which are not known to require large amounts of malonyl-CoA, suggest that further study of these tissues is in order to complete our understanding of this important enzyme system and its role in plant metabolism.
CHAPTER 3

EXPRESSION OF PLASTIDIC ACETYL-COA CARBOXYLASE

CAC1 PROMOTER:GUS FUSIONS IN DEVELOPING ARABIDOPSIS

Introduction

In plants acetyl-CoA carboxylase catalyzes the reaction of acetyl-CoA and bicarbonate to form malonyl-CoA. In the plastids of plants this reaction is the first committed step in de novo fatty acid biosynthesis. A cytosolic isozyme of ACCase produces a separate pool of malonyl-CoA that is used to elongate the fatty acids exported from the plastids for use in membranes, oils, or waxes, as well as generating a variety of secondary compounds. The two isoforms are required due to the impermeability of the subcellular membranes to malonyl-CoA. The separation of functions is also reflected, in Arabidopsis, by the existence of two ACCase forms: plastidic and cytosolic. The cytosolic ACCase consists of a homomeric protein assembled from two identical, multifunctional polypeptides. Each polypeptide contains regions related to the biotin carboxylase, biotin-carboxyl carrier, and carboxyl-transferase activities of the holoenzyme. In contrast, the plastidic form of ACCase is a heteromeric enzyme made up of four individual polypeptides, which separate the functions of the enzyme among themselves. The functions of the enzyme have been associated with each of the genes: cac2 is the biotin carboxylase, cac1 is the biotin containing carboxyl carrier, cac3 and accD make up the carboxyl-transferase function. The results of previous studies have shown that all four genes' mRNAs were expressed in a coordinated manner, increasing and decreasing over the same time in the same tissues. Expression reached its maximum level of accumulation in expanding tissues and developing
seeds. In order to further our understanding of how the plastidic ACCase genes are regulated, transgenic plants containing promoter:GUS fusions of the cac1 gene were analyzed. Because all subunits of the plastidic ACCase are coordinately expressed, this data should give insight to the general expression patterns of all the heteromeric genes. Promoters used were either a 1.1 kb fragment or a 0.5 kb fragment upstream of the cac1 gene. The expression patterns of these fusions were analyzed throughout seedling and silique development, as well as, under different stress conditions.

Materials and methods

Transformation and selection of plant material

Arabidopsis plants at the T1 generation containing the cac1 promoter:GUS transgenes were obtained from Dr. Basil Nikolau (Iowa State University). Transformation had been carried out via dipping flowering plants in a liquid culture of Agrobacterium tumefaciens (Clough and Bent 1998) containing the pBI101.2 vector plasmid (Clontech). The plasmid, in turn, contained either a 1.1 kb or a 0.5 kb cac1 upstream promoter fragment fused to the β-glucuronidase reporter gene, termed 1.1CAC and 0.5CAC respectively, and a kanamycin resistance marker. After initial selection of the T1 plants, the resistant lines were further tested to ensure homozygosity of the transgene by analysis of kanamycin resistance segregation in consecutive generations. Ultimately two lines for each construct were chosen for study.

Growth conditions

Arabidopsis thaliana, ecotype Columbia, seeds were surface sterilized for 7-10 minutes in an aqueous solution of 50% bleach (2.5% hyperchlorite) and 0.02% Triton X-100
then rinsed three times with sterile distilled water. The seeds were then placed in petri plates containing 4.3 g/L MS salts (Sigma), 1X B5 vitamins (Sigma), 10 g/L sucrose, 0.5 g/L MES, and 0.8% agar at pH 6.7 (for selection 50 µg/ml kanamycin was added) and parafilm sealed after drying. The plates were placed under constant illumination at 28°C for up to 14 days (unless otherwise specified) after which the plants were transferred to 4 inch pots containing Sunshine LC mix 1 (SunGro Horticulture) and allowed to mature with similar light and temperature conditions. After the initiation of flowering individual flowers were tagged using colored thread on the day of flowering so that they might be harvested at specified times during flower and silique development (Bowman 1994).

**Stress-inducing growth conditions**

For the purpose of testing the response of *cac1* to stress-inducing conditions, plates of seeds were prepared as described above but placed into different growing conditions and collected for GUS staining at several time points. After germinating for five days in standard conditions a cold temperature stress was induced in the seedlings by placing them in a cold room, maintained a temperature of 10°C with light provided by fluorescent bulbs at a similar intensity as the standard conditions (McKown 1996). Plants were also grown under blue light fluorescent tubes, Phillips F40 BL (350-400nm), at 65µE and 28°C to induce UV stress and anthocyanin production (Kubasek 1992, Noh 1998). As a test for light effects on *cac1* expression some plates were placed in a dark room with no or faint green light exposure before collection. Control samples were grown under standard light and temperature conditions and processed at the same time as the experimental groups to limit variations due to staining or processing variations.
Histochemical analysis of GUS activity

GUS expression was analyzed in two lines each of the 1.1CAC and 0.5CAC transgenic plants, with similar results for the lines containing identical constructs. Plant material was placed whole (seedlings and flowers) or partially dissected (siliques) into a staining solution of 30 mM NaH$_2$PO$_4$, 50 mM Na$_2$HPO$_4$, 8 mM EDTA, 0.12% Triton X-100, 4 mM K$_3$[Fe(CN)$_6$], 4 mM K$_4$[Fe(CN)$_6$] · 3H$_2$O, 20% methanol, and 1.53 mM X-Gluc (5-bromo-4-chloro-3-indoyl-(D-glucuronic acid)) (Jefferson 1987) and vacuum infiltrated for 10 minutes at -20 in. Hg. Samples were incubated at 37°C for 3 hours (germinating seeds) to 18 hours (all other samples) to allow for GUS product accumulation. Chlorophyll was cleared from stained tissue using 70% ethanol, which was also used to store samples. Fully processed samples were photographed using a dissecting stereomicroscope and Kodak Elite Chrome 160T film. The resulting slides were then computer scanned and processed using Adobe PhotoShop 5.0.2® for PowerMacs. All experiments were carried out at least twice with 10-20 samples collected and processed for each transgenic line per repetition.

Results of cac1 promoter:GUS fusion analysis

*Arabidopsis* plastidic ACCase is encoded by four individual genes that must be co-expressed to form a functional enzyme. Previous Northern blotting and *in situ* hybridization studies have indicated the mRNA accumulation of these genes is greatest in rapidly expanding portions of seedlings and flowers, as well as developing seeds prior to oil accumulation (Ke 2000). In order to investigate the gene promoter's role in this expression, transgenic plants containing a promoter:GUS fusion using a 1.1 kb fragment upstream of the *cac1* gene, termed 1.1CAC, and another containing a smaller upstream fragment of 0.5 kb,
termed 0.5CAC, were used to test how much of the promoter region was required to give the localization patterns determined by the previous studies.

**Expression of the cac1 promoter:GUS fusions in flowers and siliques**

During flower and silique development plastidic ACCase produces malonyl-CoA used for *de novo* fatty acid synthesis. Fatty acids are required for new cell membranes, cuticular waxes, and seed oils as the flower, silique and seeds mature. In early (stage 8) flower buds the expression driven by the 1.1CAC construct is predominately in the tip of the pistil (fig. 3-A). As the flower opens, GUS staining is localized to the petals and sepals, then the anthers, while remaining high in the pistil tip (fig. 3-C). When the silique and seeds are developing, 1.1CAC:GUS expression is detected at low levels in the silique walls, with staining evident in seeds that have been cut open (fig. 3-I). A different expression pattern is observed for the 0.5CAC construct. In young buds and flowers the GUS staining is more intense and widespread than that of 1.1CAC, with expression detected in petals, sepals, anthers, stigma, and ovules (fig. 3-B). Expression remains high throughout flower opening (fig. 3-D) and as the young seeds develop (fig. 3-F). Even as the silique matures, 0.5CAC driven staining is only slowly reduced in the silique walls (fig. 3-H) with completely mature seeds still staining when cut open (fig. 3-J).

**Seedling expression of the cac1 promoter:GUS constructs**

In flowers and siliques the shorter promoter of the 0.5CAC construct drives more widespread and intense GUS staining. In contrast, the 1.1CAC construct has greater expression in seedlings grown under standard conditions. Initially, both the 0.5CAC and 1.1CAC constructs express very strongly throughout the entire body of the newly germinated seedlings (fig. 4-A,B). By five days after plating, however, a difference may be observed; the
Figure 3. Cac1:GUS expression in developing flowers and siliques.

Plants containing the 1.1CAC construct and the 0.5CAC construct were labeled as flowers opened. Samples were collected at specific days after flowering (DAF), corresponding to stages of development, and stained for GUS activity. Fig (A) 1.1CAC before flower opening; (C) 1.1CAC 2 DAF; (E) 1.1CAC 5 DAF; (G) 1.1CAC 7 DAF; (I) 1.1CAC 13 DAF. Fig. (B) 0.5CAC before flower opening; (D) 0.5CAC 2 DAF; (F) 0.5CAC 5 DAF; (H) 0.5CAC 7 DAF; (J) 0.5CAC 13 DAF; Bar = 5 mm in (A-C), and (E-J); 2 mm in (D).
Figure 4. Cacl1: GUS expression in developing seedlings.

Seedlings containing the 1.1CAC (A, C, E, G) and 0.5CAC (B, D, F, H) promoter:GUS constructs were collected at specified days after plating (DAP) to follow changes in expression as the tissues grew and matured. At least ten samples per line were stained for GUS activity in each of three replications; images shown are representative of patterns obtained. Fig (A) and (B) 2 DAP; (C) and (D) 5 DAP; (E) and (F) 7 DAP; (G) and (H) 11 DAP; Bar = 500 µm in (A), and (B); 1 mm in (C-H).
shorter 0.5CAC construct expressed is in stems and cotyledons, but appears to lack any significant expression in the root of the seedling while the 1.1CAC plant stains blue in the stems, cotyledons, and roots (fig.4-D,C). Over the next nine days the difference in expression becomes more pronounced. The 1.1CAC plant shows only a slight reduction in staining of the hypocotyl and young true leaves with high levels in the root, crown, cotyledons, and initial leaves (fig. 4-E,G). The 0.5CAC construct has reduced levels of expression as the seedling grows, with little detectable staining in the root and crown region, reduced expression in the cotyledons, and less GUS staining detected in the true leaves (fig. 4-F,H) compared to the 1.1CAC plants.

**Expression of cac1 promoter:GUS transgenes in stressed seedlings**

For the purpose of testing the presence of regulatory motifs that respond to stress in the *cac1* promoter, seedlings were subjected to a variety of stresses. Alterations in growth were observed in cold and dark grown plants, but the pattern of 1.1CAC and 0.5CAC expression did not show any significant changes. Dark grown plants of the 1.1CAC line showed expression throughout the etiolated seedlings (fig. 5-D,E,F), while 0.5CAC plants lacked root expression and had relatively less staining than the 1.1CAC plants (fig. 5-N,O,P). The seedlings placed in the coldroom showed similar staining patterns to those grown under standard conditions but were delayed in growth, shown by slower increases in size and development of true leaves (fig. 5-G,I,Q,R ). Blue light was used as a photo-stress, and may have resulted in some bleaching of the plants as they grew (noticed after final collections and not shown in figure) but did not alter the basic *cac1*:GUS expression patterns (fig. 5-I,J,S,T). This reduction in photosynthetic capacity may explain the reduced leaf expansion observed in these plants.
Figure 5. Expression of *cac1*:GUS transgenes in stressed seedlings.

Plants containing the 1.1CAC (A through J) and 0.5CAC constructs (K through T) were subjected to different stresses in order to determine the response of the differing promoter constructs to environmental conditions. Seedlings were grown under the specified conditions for 5, 7, or 14 days before being processed for GUS activity. The 1.1CAC construct images: light grown seedlings: (A) 5 DAP; (B) 7 DAP; (C) 14 DAP; dark stressed seedlings: (D) 5 DAP; (E) 7 DAP; (F) 14 DAP; cold stressed seedlings: (G) 7 DAP; (H) 14 DAP; blue light stressed seedlings: (I) 7 DAP; (J) 14 DAP. Images of the 0.5CAC construct: light grown seedlings: (K) 5 DAP; (L) 7 DAP; (M) 14 DAP; dark grown seedlings: (N) 5 DAP; (O) 7 DAP; (P) 14 DAP; cold stressed seedlings: (Q) 7 DAP; (R) 14 DAP; blue light stressed seedlings: (S) 7 DAP; (T) 14 DAP. Bar = 5 mm in (A-E), (G), (I), (L), (N), and (O); 2 mm in (F), (H), (J), (K), (M), (P-S).
Discussion

Despite the central role of ACCase in de novo fatty acid synthesis in plants, this enzyme's expression and regulation is only beginning to be understood. Part of the historical difficulty in studying ACCase has been the presence of its multiple isoforms. *Arabidopsis*, for example, contains two homomeric cytosolic enzymes which produces malonyl-CoA for the elongation of fatty acids and the production of secondary compounds. It also contains an easily dissociated plastidic heteromeric isoform, whose product is the precursor to de novo fatty acids.

*Arabidopsis* plastidic ACCase is composed of peptides encoded by three nuclear genes and one plastidic gene. In order for the plant to have a ready supply of malonyl-CoA for fatty acid biosynthesis these four components must be at the right place at the right time within the plant cell. One way that this may be accomplished is through the use of common promoter activation or suppression motifs. Through the analysis of a set of promoter:GUS fusions it is possible to determine where these motifs may be located. In the case of the *cac1* 1.1CAC and 0.5CAC constructs it is clear that some elements resulting in differential expression exist upstream of the 0.5 kb fragment used in the 0.5CAC plants. The additional 0.6 kb of the 1.1CAC construct seems to reduce expression in the developing flower and silique but allows for greater expression in the seedling, particularly the root. It also seems that as none of the stresses applied to the plants changed the GUS staining patterns. By reviewing the sequence of the promoter fragments it may be possible to find a common motif between *cac1* and the other components of the heteromeric ACCase that, in turn, leads to the identification of a transcription factor capable of influencing the production of plastidic ACCase. Because of the importance of plastidic ACCase in the production of fatty acids, for
use in membranes, waxes and storage oils, the identification of a promoter region which influences expression should aid our understanding of how the plant regulates this process and provide greater molecular level understanding of the roles and origins of ACCase in plants. With this knowledge it may also be possible to engineer plants which produce economically important products or express alternative genes in known patterns for research purposes.
REFERENCES


Purification and properties of two forms of acetyl-CoA carboxylase from rat liver. J. Biol.
Chem. 263: 6447-6453.


primary structure of yeast acetyl-CoA carboxylase. Proc. Natl. Acad. Sci. USA 89:4534-
4538.

Annu. Rev. Biochem. 52:537-579

139:421-428.

differential phosphorylation of the 280-kDa component (isozyme) of rat liver acetyl-CoA

elaioplasts of the tapetum in developing Brassica anthers and their recovery on the pollen
surface. Lipids 34:517-523.

Genomic organization of 251 kDa acetyl-CoA carboxylase genes in Arabidopsis: tandem
gene duplication has made two differentially expressed isozymes. Plant Cell Physiol. 36:779-
787.