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THE BIOSYNTHESIS OF ABSCISIC ACID IN RIPENING AVOCADO FRUIT AND ETHYLENE BIOSYNTHESIS IN FERNS

By

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ABSTRACT

THE BIOSYNTHESIS OF ABSCISIC ACID IN RIPENING AVOCADO FRUIT AND ETHYLENE BIOSYNTHESIS IN FERNS

By

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Plant hormones play critical roles in growth and development. The understanding of the biosynthesis of plant hormones makes possible the manipulation of their levels, which in turn has important practical implications. The biosynthesis of two plant hormones, ethylene and abscisic acid, was the focus of this thesis.

Ethylene is a gaseous two-carbon olefin that has numerous roles such as in fruit ripening, senescence, and abscission. In higher plants, ethylene is synthesized from S-adenosyl-methionine, which in turn is converted into 1-aminocyclopropanoic acid (ACC) through the action of ACC synthase. The ACC is converted into ethylene by ACC oxidase. The pathway of ethylene biosynthesis was examined in two lower plants, the semi-aquatic ferns *Regnellidium diphyllum* Lindm. and *Marsilea quadrifolia* L. As a positive control for the ethylene biosynthetic pathway of higher plants, leaves of *Arabidopsis thaliana* (L.) Heynh. were included in each experiment. Treatments that either increased or inhibited ethylene production in *Arabidopsis thaliana* did not affect ethylene production in *Marsilea* and in *Regnellidium.* Despite the apparent differences, ACC was detected in both ferns, as was ACC synthase activity. Compared to *Arabidopsis*, leaflets of *Regnellidium* and *Marsilea* incorporated little [^{14}C] ACC and [^{14}C] methionine into [^{14}C]ethylene. From these data, it appears that formation of

ethylene in both ferns occurs mainly, if not only, via an ACC independent route, even though the capacity to synthesize ACC is present in both of these lower plants.

Abscisic acid (ABA) is a 15-carbon sesquiterpenoid derived from the oxidative cleavage of epoxycarotenoids. ABA levels increase dramatically when a wilting stress is imposed on leaves. The key regulatory step governing this increase is cleavage from carotenoids. The gene encoding the 9-*cis*-epoxycarotenoid dioxygenase (NCED) that catalyzes this cleavage reaction was first cloned from a *viviparous* mutant of maize, *vp14*.

To address whether the developmental increase in ABA level that occurs during avocado fruit ripening is governed by oxidative cleavage from carotenoids, three *Vp14* homologs were cloned from ripening avocado fruit. Two of these homologs, *PaNCED1* and *PaNCED3*, are approximately 60% identical at the amino acid level to *Vp14*, and increase in expression during fruit ripening. When expressed as recombinant proteins, both PaNCED1 and PaNCED3 could catalyze the cleavage of 9-*cis*-violaxanthin and 9-*cis*-neoxanthin into xanthoxin. A third *Vp14* homolog cloned from avocado fruit, *PaNCED2*, is 30% identical at the amino acid level to *Vp14*, and is constitutively expressed during both fruit ripening and during wilting of leaves.

Hormone levels are determined by both their rates of synthesis and degradation. ABA is metabolized by a cytochrome P450 monooxygenase, ABA-8'-hydroxylase, into phaseic acid. As it is been demonstrated that the induction of gene encoding ABA-8'-hydroxylase occurs at a transcriptional level, a modified differential display approach was used to isolate cytochrome P450 monooxygenase gene that may encode ABA 8' hydroxylase.

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Chapter 1

General Introduction

Plant hormones are natural compounds that play essential roles in growth and development (Kende and Zeevaart, 1997). The term plant hormone was originally derived from animal physiology to denote a chemical messenger. The following criteria must be met if a compound is to be designated a plant hormone: 1) localized site of synthesis within the plant, 2) active at low concentrations, and 3) controls a physiological response via the concentration of the hormone (Davies, 1995). In addition to the five classical plant hormones (auxin, cytokinin, ethylene, gibberrellin, and abscisic acid), there are now three additional plant hormones: brassinosteroids, oligosaccharins, and jasmonates (Creelman and Mullet, 1997).

The practical applications that can be derived from knowledge of the pathways of biosynthesis, the perception, and the interactions between plant hormones are numerous. For example, millions of dollars are lost as a result of spoilage of fruit that is attributable to ethylene production. Tomatoes are treated with ethylene following picking to allow ripening to occur. Unfortunately, the flavor seldom matches that which develops when the fruit is allowed to ripen on the vine. The first steps towards developing decreases in fruit spoilage would be understanding how ethylene levels are regulated inside the plant, how ethylene is perceived, and how ethylene causes changes in gene expression. In fact, knowledge of the ethylene biosynthetic pathway allowed for the creation of antisense tomato fruits that fail to ripen in the absence of added ethylene (Oeller et al., 1991).

Another example of a plant hormone that plays a critical role in a problem of practical importance is abscisic acid (ABA). Much of the world suffers from drought

stress and salinization. This water stress limits productivity of crops, and hence has a direct impact on global food production (Boyer, 1982). The plant hormone considered most involved in adaptation to this osmotic stress is ABA. As most plants suffer from drought stress at some point in their life cycle, they have developed various adaptive mechanisms that ensure survival under these conditions. Some of these adaptations include the synthesis of unique proteins with unknown functions such as LEA proteins (Baker et al., 1988), the synthesis of which is induced by ABA. ABA also functions directly by mediating closure of stomata, thus preventing water loss through transpiration. An understanding of how ABA levels increase during drought stress, and how gene expression is activated in response to the increased ABA, is critical for the improvement of drought-tolerant properties in plants.

This thesis focuses on the biosynthesis of two of the "classical" plant hormones, ABA and ethylene.

Ethylene Biosynthesis

Ethylene is a two-carbon gaseous plant hormone that plays a role in processes such as senescence, ripening of climacteric fruit, and elongation of some aquatic plants such as rice (reviewed by Abeles et al., 1992). In etiolated pea seedlings, ethylene has three major effects, the so-called "triple response": 1) diageotropic growth, 2) thickening of the stem and inhibition of stem elongation, and 3) exaggeration of apical hook curvature. These effects served as a basis for various genetic screens used to identify several classes of ethylene response mutants (discussed later). Ethylene is synthesized via the Yang cycle, shown in Figure 1.1. S-adenosyl-methionine is converted through the action of ACC synthase to 1-aminocyclopropane-1-carboxylic acid (ACC)

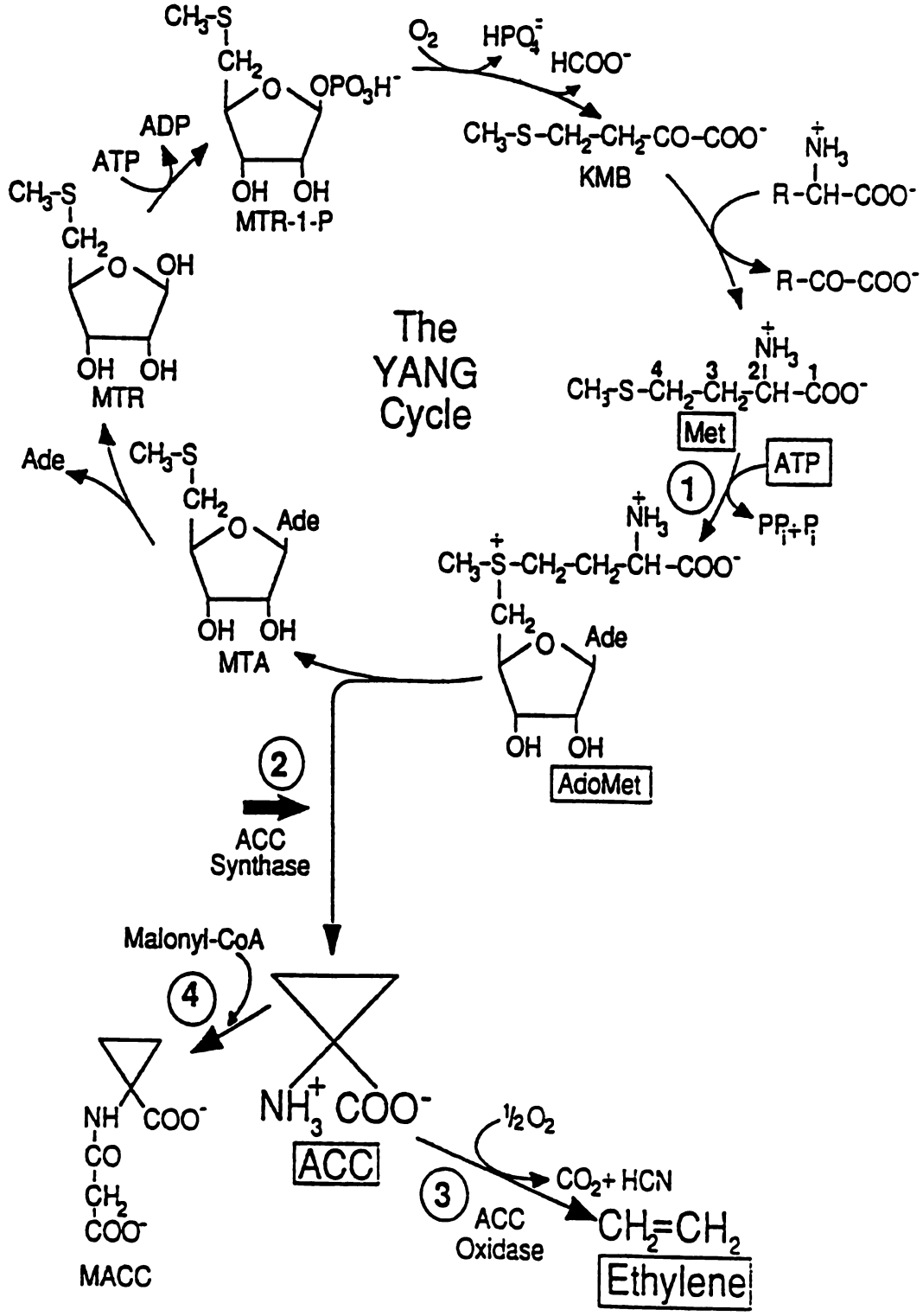

Figure 1.1. Ethylene biosynthetic pathway in higher plants (Taken from Zarembinski and Theologis (1994).

The ACC is oxidized to ethylene by ACC oxidase. A major breakthrough in the elucidation of the pathway occurred in 1979, when 1-aminocyclopropane-1-carboxylate (ACC) was discovered as the immediate precursor to ethylene (Adams and Yang, 1979). Apple fruit treated with [^{14}C]-methionine under anaerobic conditions were blocked in ethylene synthesis but accumulated labeled ACC. When returned to air, the labeled ACC was rapidly converted into ethylene. Enzyme activity of ACC synthase was first obtained from tomato by Boller et al. (1979), and was shown to require pyridoxal phosphate. The pyridoxal phosphate-dependency of the enzyme was utilized as a means of identifying the active site peptide (Yip et al., 1990).

To date, numerous ACC synthase genes have been cloned (Johnson and Ecker, 1998), and various ACC synthases have been biochemically purified from sources such as wounded and ripe tomato fruit, wounded winter squash, IAA-induced zucchini fruit, and ripe apple (Kende, 1993). ACC synthase genes share seven blocks of high homology. One such block is the active site region, present in all pyridoxal-phosphate dependent enzymes (Kende, 1993). In plant species examined, ACC synthase is encoded by a multi-gene family whose members are differentially regulated. Some of the conditions known to induce ACC synthase genes are ripening, senescence, germination, wounding, water-logging, chilling, and drought. From this it can be concluded that ACC synthase plays a critical role in regulating ethylene synthesis under a variety of conditions.

The gene encoding the second enzyme in the ethylene biosynthetic pathway, ACC oxidase, was first isolated from tomato by differential screening of a tomato ripening-related library. Antisense expression of a cDNA of unknown function (pTOM13) reduced ethylene formation and ACC oxidase activity in transgenic tomato

plants (Hamilton et al., 1990). Confirmation of the role of pTOM13 was verified by *in vitro* expression in two heterologous systems, *Xenopus* oocytes (Spanu et al., 1991), and in yeast (Hamilton et al., 1991). The derived protein sequence of pTOM13 was similar to ascorbate-dependent dioxygenases. From the sequence similarity, an *in vitro* assay was developed for the conversion of ACC into ethylene (Ververidis and John, 1991). Like other α-ketoglutarate dependent enzymes, ACC oxidase has a requirement for Fe^{2+} and ascorbate. However, unlike other dioxygenases, ACC oxidase has an additional requirement for CO_2 and does not require α-ketoglutarate as a co-substrate (Prescott, 1993).

Numerous ACC oxidase genes have been cloned from a variety of sources (Johnson and Ecker, 1998). Similar to ACC synthase, ACC oxidase is encoded by a small multi-gene family in many plant species. In some vegetative tissues, ACC oxidase is constitutively expressed, but in tomato the ACC oxidase genes exhibit tissue and developmental differences in expression (Barry et al., 1996). ACC oxidase also participates in a feedback loop in which ethylene alters the activity of both ACC synthase and ACC oxidase (Kende and Zeevaart, 1997).

Ethylene Signal Transduction

The first genetic screens to identify ethylene-responsive mutants utilized etiolated *Arabidopsis* mutants (*etr* mutants) that either failed to show the triple response (Bleecker et al., 1988), or that constitutively expressed the ethylene-induced phenotype (*ctr* mutants) (Kieber et al., 1993). Mutant *etr* seedlings are tall and have an open hook in comparison to wild-type plants. The *ETR* gene (for Ethylene Triple Response) was cloned, and the deduced protein sequence encodes a two-component receptor (Chang et

al., 1993). Schaller and Bleecker (1995) confirmed the role of ETR1 in binding ethylene by heterologous expression in yeast. The *CTR1* gene encodes a protein with homology to the Raf type-serine/threonine protein kinases from mammals, indicating that the ethylene signal transduction pathway could feed into a MAP kinase pathway. Several homologs of *ETR1* have been cloned. These include *ETR2*, *EIN4* (ethylene-insenstive), and *ERS*, all of which confer dominant ethylene insensitivity to *Arabidopsis* seedlings. In addition, many homologs of *ETR1* and *ERS* have been isolated from tomato. Thus, it appears that ethylene perception, like ethylene biosynthesis, is genetically redundant (Johnson and Ecker, 1998).

Subsequent genetic analysis has revealed that CTR1 acts downstream of ETR1, and that it likely regulates a family of transcription factors (the EIN3 family). Other components of the signal transduction pathway have also been identified and their function is under investigation. As a result of such genetic analysis, a detailed picture of the ethylene signal transduction pathway is now emerging (Johnson and Ecker, 1998).

Background of the project

While the ethylene biosynthetic pathway in higher plants has been known for some time, there has been relatively little investigation into the pathway in lower plants. This question becomes important in light of the fact that bacteria and fungi use α-ketoglutarate instead of methionine as a precursor. Since α-ketoglutarate is a cofactor in the higher-plant pathway, it is possible that the higher-plant pathway has evolved from the bacterial pathway by a change in enzyme substrate specificity. Lower plants, like mosses and ferns, conceivably could have a combination of both pathways. A report in the literature published prior to biochemical knowledge of the higher-plant pathway

described the insensitivity of ethylene production in *Marsilea quadrifolia* and *Regnellidium diphylllum* to inhibitors of the higher-plant pathway (Cookson and Osborne, 1978). The ethylene biosynthetic pathway in these two semi-aquatic ferns was therefore re-investigated in view of the current knowledge of the higher-plant pathway.

Abscisic Acid Biosynthesis

Abscisic acid was originally named abscisin II because of its proposed role in leaf abscission. Since that time, ABA has been shown to play a role in many processes in plant growth and development. ABA is involved in responses to cold, salt, and drought, and in functions related to seed germination and embryo development (Zeevaart and Creelman, 1988). Evidence for the involvement of ABA in these processes is provided by: 1) Various ABA-deficient mutants that have reduced ABA content and are altered in various responses (Koornneef et al., 1998), 2) Correlations between endogenous ABA levels and various physiological/developmental processes, 3) The effects that added ABA has on gene expression and on restoring phenotypes of various mutants (see Leung and Giraudat, 1998).

Synthesis of the sesquiterpenoid ABA occurs through an indirect pathway in which a 40-carbon carotenoid precursor is cleaved to the 15-carbon compound xanthoxin (XAN) that is subsequently be converted into ABA (Figure 1.2). The use of mutants has been instrumental in the deduction of the ABA biosynthetic pathway (Koornneef et al., 1998). Firstly, these mutants provided evidence in favor of the indirect pathway and secondly, uncovered the order of specific steps within this pathway (see for example, Schwartz et al., 1997a). Evidence that favors the indirect pathway from xanthophylls is as follows: 1) The carotenoid biosynthesis inhibitors fluridone and norflurazon also inhibit

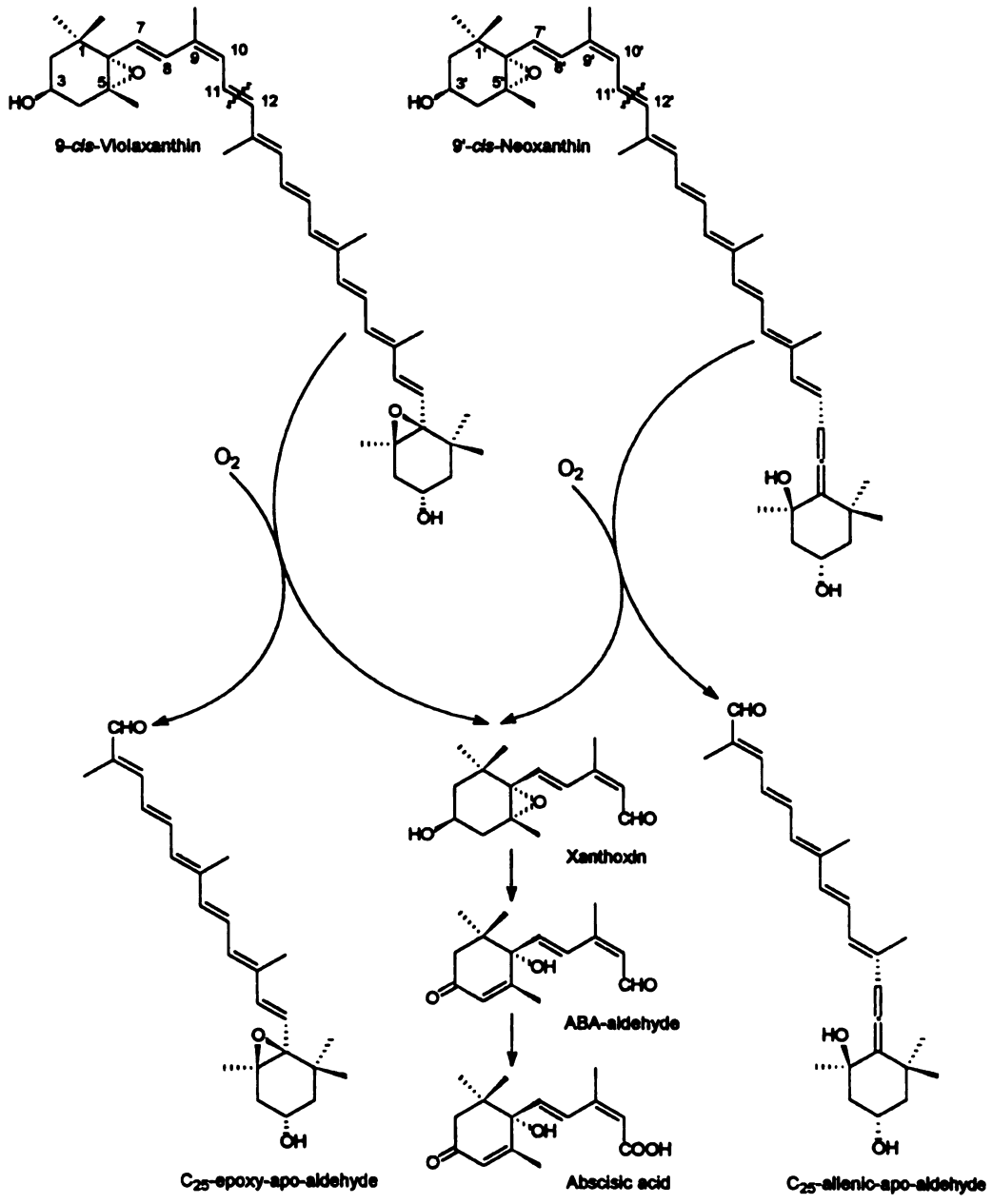

Figure 1.2. Proposed ABA biosynthetic pathway in higher plants. The cleavage of 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin into xanthoxin requires oxygen and ferrous iron. The enzymes converting xanthoxin into ABA-aldehyde and ABA-aldehyde into ABA are thought to be constitutive in most plant tissues, and hence, are not likely to be key regulatory steps. (Taken from Schwartz et al., 1997b)

ABA biosynthesis (Gamble and Mullet, 1986), 2) Viviparous mutants of maize blocked in early stages of carotenoid biosynthesis are ABA-deficient (Neill et al., 1986), 3) ^{18}O-labeling experiments with water-stressed leaves demonstrated little incorporation of ^{18}O onto the ring but incorporation into the side chain, indicating that a large precursor pool already containing oxygen on the ring exists (Zeevaart et al., 1989), 4) In both water-stressed roots and dark-grown water-stressed leaves, a 1:1 molar correlation between a decrease in *trans*-violaxanthin and 9'-*cis*-neoxanthin and an increase in ABA and its metabolites was observed (Li and Walton, 1990, Parry et al., 1990, Parry et al., 1992). Due to the weight of this evidence in conjunction with more recent biochemical and molecular data (Schwartz et al., 1997b; Tan et al., 1997), the direct pathway (wherein farnesyl pyrophoshate would serve as a precursor) has been excluded as an ABA source in plants.

The experiments that provided proof for the indirect pathway also provided evidence to support the regulatory nature of xanthophyll cleavage in increasing ABA levels. The ^{18}O-labeling experiments showed that a large precursor pool size exists, and that only small changes in the pool size would be needed to account for the increases in ABA that occur in response to wilting of leaves. The immediate product of the cleavage reaction, XAN, is readily converted into ABA both *in vivo* and *in vitro* (Sindhu and Walton, 1987). In addition, the enzymes that convert XAN to ABA-aldehyde and ABA-aldehyde into ABA are constitutively expressed (Sindhu and Walton, 1988). Therefore, these steps are not rate-limiting for ABA biosynthesis. Further, zeaxanthin epoxidase, the enzyme that converts zeaxanthin into violaxanthin, is not induced during wilting of tomato leaves (Burbidge et al., 1997). Taken together, the data indicate that another

enzyme within the biosynthetic pathway must be regulatory.

Feeding experiments have demonstrated that XAN can be converted to ABA whereas *trans*-XAN cannot. Therefore, xanthophylls must be in the 9-*cis*-configuration in order to serve as *in vivo* precursors to ABA. In leaves, the bulk of violaxanthin is in the all-*trans* form; the all-*cis* form is a minor component. This suggests that *in vivo*, neoxanthin may be the ABA precursor, because it is present mainly in the 9-*cis* form (Zeevaart, 1999). Non-green tissues can be classified into four types with regard to their neoxanthin composition: only all-*trans* present, only 9'-*cis* present, neither present, or a combination of the two present (Takaichi and Mimuro, 1998). Thus, in some tissues, the question of the isomerization reaction may be relevant. In addition, the configuration of the xanthophylls within the membrane may also be important in dictating the *in vivo* precursor of ABA.

Because of the presumed lability of the carotenoid precursors, and the likely membrane localization of the xanthophyll cleavage enzyme, demonstration of enzymatic activity of C_{40}- carotenoids into XAN has been problematic. A combined molecular, biochemical and genetic approach led to the elucidation of the cleavage reaction. A transposon-tagged mutant of maize, *viviparous 14* (*vp14*), exhibits defects in ABA synthesis in both embryos and in water-stressed leaves (Tan et al., 1997). The deduced amino acid sequence of *Vp14* is similar to lignostilbene dioxygenases. Recombinant VP14 catalyzes the cleavage of 9-*cis*-xanthophylls into XAN and a C_{25}- apoaldehyde. The stoichiometric relationship between the products formed indicated that the cleavage was non-random, and that it occurred between the 11 and 12 position of the polyene chain (Schwartz et al., 1997b). Various genes with homology to *Vp14* have appeared in

the database. Collectively, these genes have been designated nine-*cis*-epoxycarotenoid dioxygenase (*NCED*) genes.

ABA Signal Transduction

There are generally considered to be two types of receptors for ABA, one of which acts extracellularly and one of which acts intracellularly. These two receptors have been found to exist in both barley aleurone cells (Bethke et al., 1997) and in guard cells (Anderson et al., 1994; Schwartz et al., 1994). While the ABA receptors remain to be discovered, considerable progress has been made in elucidating components within the signal transduction pathway leading to ABA-induced gene expression (Leung and Giraudat, 1998). Most of these components have been found through various mutant screens, although others have been identified through microinjection experiments (Wu et al., 1997), and biochemical approaches (Ritchie and Gilroy, 1998).

Mutants in ABA responsiveness have been isolated mainly by screening for plants that have altered germination characteristics and seedling growth, and cannot be rescued by exogenous ABA (Koornneef et al., 1998). The maize *viviparous 1* (*vp1*) mutant is viviparous as a result of altered sensitivity to ABA. The *Vp1* gene has been cloned and encodes a transcription factor that confers promoter hypersensitivity to ABA. Various *Arabidopsis thaliana* ABA-insensitive loci (*ABI1,2,3*) have been identified by selecting seeds capable of germinating on ABA levels that are inhibitory in wild-type. The *ABI3* gene is an ortholog of *Vp1* (Giraudat et al., 1992), while *ABI1* and *ABA2* encode homologs of serine/threonine phosphatase 2C (Leung et al., 1997). The *ERA1* to *ERA3* (Enhanced Response to ABA) loci were identified by lack of seed germination in the presence of low concentrations of ABA that are not inhibitory to the wild-type. The

ERA1 gene encodes a β-subunit of farnesyl transferase that may function as a negative regulator of ABA signaling (Cutler et al., 1996).

Using microinjection into tomato hypocotyl cells, Wu et al. (1997) have identified cyclic ADP-ribose as a signaling molecule in the ABA response. Cyclic ADP-ribose exerts its effect by way of calcium. Ritchie and Gilroy (1998) reported that ABA-mediated inhibition of gibberellin-stimulated responses is dependent upon the activation of phospholipase D in barley aleurone cells. This suggests that phosphatidic acid is also involved in ABA signaling.

Functional dissection of ABA-responsive promoters identified *cis*-acting elements involved in ABA-induced gene expression (Shinozaki and Yamaguchi-Shinozaki, 1997). The ABREs (ABA Response Elements) are present in *Rab16 LEA* genes of rice, the *Em LEA* gene of wheat and the *C1* gene (although in the latter case, the element may not be the major determinant of ABA responsiveness). In other cases, there are some *cis*-elements that are responsive to osmotic stress but not to ABA, e.g. Dehydration Response Elements (DRE) in *Rd29A*. Specific proteins that bind to these elements have been identified (Zhu et al., 1997). The combined results from the identification of DNA-binding factors and from the identification of intermediates within the ABA signaling pathway will eventually form a comprehensive view of ABA signal transduction. Of particular interest is the overlap between ABA-mediated stress responses and other stresses, such as salt and cold. Also, the existence of cross-talk between the developmental signaling by ABA and stress-related signaling is intriguing (Zhu et al., 1997).

Objective of the Project

As mentioned, ABA plays a role in both physiological and developmental processes. Water stress-induced increases in ABA levels are likely regulated at the level of cleavage of epoxycarotenoids into XAN. The objective of the work presented in Chapter Three was to determine whether the same regulatory controls on ABA biosynthesis exist in fruit as in leaves. Avocado fruit was chosen as it exhibits large increases in ABA levels as ripening occurs (Zeevaart et al., 1989). Homologs of *Vp14* were cloned from avocado fruit and their expression, localization, and *in vitro* function determined. Changes in carotenoid levels in fruit were also determined to see if correlations could be made between *in vivo* increases in ABA and losses of specific carotenoids.

ABA Catabolism

As with any substance, ABA levels are controlled at both the levels of synthesis and degradation. The pathway of ABA inactivation varies depending on the plant species, tissue, and developmental stage (Zeevaart, 1999). The two major pathways used are 1) conjugation into glucose-esters or alcohols, and 2) oxidation to form phaseic acid (PA) (Zeevaart, 1999). Oxidation of the 8' methyl group of ABA yields 8'-OH-ABA, which is unstable and rearranges to form PA.

Leaves subjected to rehydration after water-stress exhibit a rapid decline in ABA levels (Zeevaart, 1980, 1983; Pierce and Raschke, 1981), with an accompanying increase in PA and later in DPA. Evidence in the literature suggests that the enzyme that carries out the conversion of ABA into PA is a cytochrome P450 monooxygenase. This evidence can be summarized as follows: 1) Addition of the cytochrome P450 inhibitor tetcyclacis

to *Xanthium* leaves prior to rehydration prevents PA accumulation (Zeevaart et al., 1990), 2) A cell-free system of *Echinocystis lobata* that is capable of converting ABA into PA was inhibited by carbon monoxide, and requires oxygen and NADPH (Gillard and Walton, 1976), 3) Maize suspension cultures catalyze the conversion of ABA into PA. The reaction requires oxygen and NADPH, and is inhibited by carbon monoxide. The carbon monoxide inhibition can be reversed by irradiation with blue light (Krochko et al., 1998).

Many plant cytochrome P450s are controlled at the level of transcription (Schuler, 1996). For example, the P450 genes within the phenylpropanoid pathway are coordinately regulated by light, fungal elicitors, development, and wounding (Bolwell et al., 1994). Uknes and Ho (1984) found that induction of ABA 8'-hydroxylase was inhibited by cordycepin and cycloheximide, indicating that the increased activity is due to transcriptional activation. In addition to various abiotic inducers known to up-regulate P450s, many cytochrome P450s are activated by their substrates. ABA has been shown to induce ABA 8'- hydroxylase (Uknes and Ho, 1984; Babiano, 1995; Cutler et al., 1997; Windsor and Zeevaart, 1997).

Objective of the Project

The fact that ABA 8'-hydroxylase is a transcriptionally regulated cytochrome P450 monooxygenase that is induced by its own substrate suggested a strategy to isolate the gene encoding this enzyme. Differential display has become a widely used and useful strategy to obtain low abundance messages that are differentially expressed (Liang et al., 1995). The procedure is basically a modified reverse-transcription PCR employing an anchored oligo d(T) primer and an arbitrary primer. Based on an alignment of

cytochrome P450s, three conserved regions were chosen for the design of degenerate primers. These degenerate primers were used in place of the arbitrary primers normally used in differential display to amplify cytochrome P450 monooxygenase genes that are up-regulated in response to ABA treatment of suspension cultures. Cytochrome P450 monooxygenase genes that are induced by ABA treatment of cultures are considered potential candidates for ABA 8'-hydroxylase.

Overall Objective

The main questions addressed in this thesis are: 1) Is there a different ethylene biosynthetic pathway utilized by ferns in comparison to that in higher plants? 2) How is the increase in ABA levels that occurs during fruit ripening regulated? and 3) Can a modified differential display approach be effectively utilized to isolate specific classes of differentially expressed genes? The knowledge gained from the study of both the biosynthesis and degradation of ABA is important in understanding how overall ABA levels in a plant are controlled. Secondly, the evolution of biochemical pathways is an interesting biological question, and has implications for how new enzymatic functions are derived.

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Chapter 2

Investigation of the ethylene biosynthetic pathway of the semi-aquatic ferns *Marsilea quadrifolia* and *Regnellidium diphyllum*

ABSTRACT

The pathway of ethylene biosynthesis was examined in two lower plants, the semi-aquatic ferns *Regnellidium diphyllum* Lindm. and *Marsilea quadrifolia* L. As a positive control for the ethylene biosynthetic pathway of higher plants, leaves of *Arabidopsis thaliana* (L.) Heynh. were included in each experiment. Ethylene production by *Regnellidium* and *Marsilea* was not increased by treatment of leaflets with 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene in higher plants. Similarly, ethylene production was not inhibited by application of aminoethoxyvinylglycine and α-aminoisobutyric acid, which are inhibitors of the ethylene biosynthetic enzymes ACC synthase and ACC oxidase, respectively. However, ACC was present in both ferns, as was ACC synthase. Compared to leaves of *Arabidopsis*, leaflets of *Regnellidium* and *Marsilea* incorporated little [^{14}C]ACC and [^{14}C]methionine into [^{14}C]ethylene. From these data, it appears that the formation of ethylene in both ferns occurs mainly, if not exclusively, via an ACC-independent route, even though the capacity to synthesize ACC is present in these lower plants. Results from this work have been published previously (Chernys and Kende, 1996).

INTRODUCTION

In higher plants, ethylene is formed via the following biosynthetic pathway: L-methionine → S-adenosyl-L-methionine (AdoMet) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene (Adams and Yang, 1979). The conversion of AdoMet to ACC and of ACC to ethylene is mediated by ACC synthase and ACC oxidase, respectively (for a review, see Kende, 1993). ACC synthase is a pyridoxal phosphate-dependent enzyme related to aminotransferases. It has been purified from a number of tissues, and numerous genes encoding ACC synthase have been cloned (Zarembinski and Theologis, 1994). ACC synthase is encoded by a multi-gene family in all plants that have been examined, and the members within the gene family are often differentially regulated in response to auxin treatment, wounding, and a variety of other stimuli (Kende, 1993). Sequence similarity of individual ACC synthase isoforms across species is often greater than within a species (Zarembinski and Theologis, 1994). ACC oxidase is related to dioxygenases, however, it differs from other dioxygenases in that it requires CO_2 as a cofactor, presumably being required for activation. Further, dioxygenases utilize α-ketoglutarate as a co-substrate by reducing it to succinate, whereas ACC oxidase does not require α-ketoglutarate for activity (Prescott, 1993).

Many semi-aquatic plants possess the capacity to elongate upon submergence (Ridge, 1987). In many cases, this response is mediated by ethylene and also requires either auxin or gibberellin. *Regnellidium diphyllum* is a semi-aquatic fern that undergoes ethylene-mediated elongation when submerged (Musgrave and Walters, 1974; Cookson and Osborne, 1978). Elongation of *Marsilea quadrifolia* is also promoted by submergence (Karsten, 1888, quoted by Cookson and Osborne, 1978), but the

involvement of ethylene in this process has not been demonstrated with this fern. Cookson and Osborne (1978) also showed that the pathway of ethylene biosynthesis in *Regnellidium* may be different from that of higher plants. Their report was published prior to the full elucidation of the ethylene-biosynthetic pathway in higher plants and did not deal with the production of ACC and the enzymes of the ACC-dependent pathway. Because of the physiological response of *Regnellidium* to ethylene and the possible evolutionary implications of an alternative ethylene-biosynthetic pathway in lower plants, we have re-investigated ethylene biosynthesis in ferns. Our results show that both ACC synthase and ACC are present in *Marsilea* and *Regnellidium*. However, the capacity to convert ACC to ethylene is either lacking or present at low levels. Thus, a major part or all of the ethylene produced by these two ferns appears to be synthesized via an ACC-independent pathway.

Studies done with aquatic plants, such as deepwater rice have shown that ethylene can mimic the effect of submergence on growth. A series of experiments were conducted to investigate whether the submergence response differed between *Regnellidium* and higher plants. The results demonstrated that *Regnellidium* exhibits a similar elongation response as higher plants, and this may serve as a useful condition for further studies on the ethylene biosynthetic pathway in this fern. Some of the results presented here have been published previously (Chernys and Kende, 1996).

MATERIALS AND METHODS

Plant material and growth conditions.

Regnellidium diphyllum Lindm. was purchased from Maryland Aquatic Nurseries (Jarretsville, MD, USA). *Marsilea quadrifolia* L. was obtained from the Botany

Greenhouse Collection at Michigan State University. Both ferns were grown in pots containing wet soil under greenhouse conditions with supplementary light. For most experiments, leaflets were removed from fronds between 5 and 10 cm in height and with newly expanded leaflets. As control, *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) was used throughout the study. Plants were grown under a photoperiod of 16 h light (250 μmol m^{-2} s^{-1}) and 8 h dark at 20 to 23°C. Lower leaves from 6- to 8-week-old plants were used in the experiments.

Application of test substances.

Leaflet discs, 1 cm in diameter, were cut from fronds of either *Marsilea* or *Regnellidium*, and about eight discs were placed, abaxial surface down, on filter paper that had been moistened with a solution of the inhibitor or water as control inside 25-ml Erlenmeyer flasks. In the case of *Arabidopsis*, two or three leaves were placed, abaxial surface down, on filter paper. The flasks were then sealed, and 1 ml of the gas phase was removed at 4-h intervals for ethylene determination by gas chromatography. To study the requirement for iron, the leaf discs or leaves were incubated with 1,10-phenanthroline. To test for reversal of inhibition by 1,10- phenanthroline, the leaves or leaf discs were transferred into flasks with 0.2 M $FeSO_4$. Aminoethoxyvinylglycine (AVG) was a gift of Hoffmann-LaRoche (Nutley, NJ, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Radiolabeling.

Leaflet discs were placed, abaxial surface down, on filter paper moistened with 74 kBq of [U-^{14}C]methionine (9.5 GBq/mmol, New England Nuclear, Boston, Mass., USA) or 37 kBq of [2,3-^{14}C]ACC (3 GBq/mmol, CEN Saclay, Gif-sur-Yvette, France) in 1 ml

of distilled water. Ethylene was allowed to accumulate for 4 h, after which time 1 ml of the gas phase was removed to determine the ethylene concentration. The gas inside the flask was removed using a 60-ml syringe while being replaced with saturated $(NH_4)_2SO_4$ to allow complete removal by preventing formation of a vacuum inside the flask, or with air if the tissue was used for ACC analysis. The syringe was immediately capped, a 1-ml gas sample was removed to determine losses of ethylene, and 0.4 ml of 0.2 M mercuric acetate in methanol was added to adsorb the ethylene. In the case of ACC assays, the gas in the vial was removed with a 10-ml syringe, and 0.2 ml of 0.2 M mercuric acetate in methanol was added to the syringe. The syringes were shaken in the cold for 2 h, following which the mercuric acetate was transferred to a scintillation vial to which 10 ml of scintillation fluid had been added. The yellow coloration caused by mercuric acetate was eliminated by adding a few drops of glacial acetic acid. The radioactivity was determined using a liquid scintillation counter.

ACC determination and thin-layer chromatography.

Approximately 1 g FW of leaflets was frozen in liquid nitrogen and ground using a mortar and pestle. ACC was extracted in 100 mM Na-phosphate buffer, pH 11.5, and the homogenate was centrifuged at 10,000g for 15 min. The supernatant was used directly for ACC determination according to Lizada and Yang (1979). To confirm that ACC was, in fact, present, leaflets from *Marsilea* and *Regnellidium* and leaves from *Arabidopsis* were frozen in liquid nitrogen and extracted with 80% methanol (2 ml/g FW). The extracts were centrifuged at 10,000g for 15 min. The supernatant was evaporated to dryness, and the resulting residue was resuspended in 50 µl 80% methanol. Ten to 20 µl of each extract were chromatographed on 0.2-mm cellulose glass plates

using *n*-butanol:acetic acid:water (60:15:30 v/v/v) as solvent (Boller et al., 1979). The plates were divided into 1- or 1.5-cm zones that were removed and eluted with 100 mM Na-phosphate buffer, pH 11. The eluate was assayed for ACC according to Lizada and Yang (1979). Standard ACC (0.1 μmol) was chromatographed in parallel with the extracts, and, after removal of the zones with samples, the remaining part of the plates was sprayed with ninhydrin reagent to determine the R_f of ACC.

ACC synthase assay.

Approximately 2 to 3 g FW of leaflet tissue was ground in liquid nitrogen and extracted with 1 ml/g FW of buffer (100 mM Na-phosphate, pH 8.0, 5 mM DTT, 10 μM pyridoxal 5-phosphate). The slurry was centrifuged at 15,000g for 20 min at 4°C. The supernatant was dialyzed overnight against two changes of buffer (10 mM Na-phosphate, pH 8.0, 5 μM pyridoxal 5-phosphate) at 4°C. The extract was concentrated to ca. 1/5 to 1/3 its original volume by placing the dialysis bag in dry polyethylene glycol 8000. For the assay of ACC synthase activity, 0.6 ml of the extract was combined with 100 μl of 1 M Na-phosphate, pH 8.0, and either 100 μl of 1 mM S-adenosyl-methionine (AdoMet) or H_2O in a 9-ml tube, and the reaction mixture was shaken at 30°C for 1 h. The ACC produced was determined by the method of Lizada and Yang (1979).

Submergence treatment.

Regnellidium diphyllum plants with newly expanded leaflets, petiole lengths between 3 and 5 cm and containing a 2 cm portion of the rhizome, were used. A 2-g weight was attached to the rhizome, and the plant submerged in a 1 l jar such that the leaflets were below the water surface. Leaflets from these plants were used for ethylene determination as described above. For ethylene treatment experiments, the plant with a

weight attached was suspended in a 1 l beaker such that the leaflets were held above the water surface. The beaker was placed inside a dessicator into which ethylene was injected. After each day, the dessicator was aerated and the ethylene replaced. Water was refilled as required. Leaflets from these plants were used for ethylene determination as described above.

Cloning of a fragment of ACC synthase from Marsilea.

For RNA isolation, fresh leaflets were frozen in liquid nitrogen and ground to a fine powder using a morter and pestle. Frozen tissue was added to extraction powder [4 M guanidine thiocyanate, 1 M ammonium thiocyanate, 3 M sodium acetate, 0.5 % polyvinylpyrrolidine (MW 360, 000), 1% β-mercaptoethanol, 0.5% laurylsarcosine], and the homogenate placed at 65°C for 30 min. The homogenate was centrifuged at 10,000g for 10 min, and the supernatant extracted with chloroform/isoamyl alcohol (24:1). The top phase was removed and two volumes of absolute ethanol were added. The nucleic acids were centrifuged at 10,000g for 10 min, and the pellet washed twice with 80% ethanol. The pellet was dissolved in cetyltrimethylammoniumbromide (CTAB) Buffer [1% CTAB, 10 mM EDTA·Na_2, 0.70 M NaCl, 0.5% polyvinylpyrrolidine (MW 360,000), 1% β-mercaptopethanol], and heated at 65° C to assist in resuspension. Undissolved material was spun down at 10,000g for 10 min, and the supernatant extracted with $CHCl_3$/isoamyl alcohol. The top phase was transferred to a clean tube and 1/10th volume of 10% CTAB (0.7 M NaCl, 10% CTAB) was added. The mixture was again extracted with $CHCl_3$/isoamyl alcohol, and two volumes of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl pH 7, 10 mM EDTA), was added to the supernatant. After 30 min at room temperature, the solution was spun (10,000g for 10 min), and the

pellet washed with 80% ethanol and resuspended in water. Reverse transcription (RT) was carried out on 4 μg total RNA using an oligo d(T) primer and MMLV reverse transcriptase (Gibco-BRL). The procedure followed was the same as described in the MMLV reverse transcriptase instruction manual. Five μl of the 20 μl RT reaction was used in PCR amplification. Degenerate primers HK62 (CTC GAA TTC ACC AAY CCN TCA AAY CCN YTR GG) and HK63 (CTC AAG CTT ACN ARN CCR AAR CTY GAC AT) designed against block 4 and block 6, respectively, of ACC synthase (Kende, 1993) and containing *Eco*R1 (HK62) and *Hind*III (HK63) at the ends of each the primers were used in the amplification. Conditions used for the PCR reaction were as follows: 94 1 min, 40 1 min, 72 45 sec for 35 cycles. The PCR product was purified from an agarose gel, digested with *Hin*dII and *Eco*R1, and ligated into pBluescript.

RESULTS

Effect of exogenous ACC (in vivo ACC oxidase activity).

As a first test whether the biosynthetic pathway in ferns differs from that in higher plants, ACC was applied to leaflet discs of both *Marsilea* and *Regnellidium*, and ethylene production was monitored over a 24-h period. Because wounding did not increase ethylene production in either *Marsilea* or *Regnellidium* (results not shown), leaflet discs were used throughout the study to improve the uptake of ACC or other test compounds. Leaves of *Arabidopsis*, which produce ethylene via the ACC pathway, were used as a positive control in all experiments. Addition of 0.1 mM ACC increased ethylene production in *Arabidopsis* while concentrations of up to 1 mM had little, if any, effect in *Marsilea* and in *Regnellidium* (Figure 2.1; see also Table 2.2).

Effect of inhibitors of the higher-plant ethylene biosynthetic pathway.

Substances that are known to inhibit ethylene production in higher plants were tested for their effect on ethylene synthesis in *Marsilea* and *Regnellidium*. Aminoethoxyvinylglycine (AVG), a potent inhibitor of pyridoxal phosphate-mediated enzyme reactions, such as catalyzed by ACC synthase (Boller et al., 1979), strongly inhibited ethylene formation in *Arabidopsis* when applied at 0.1 mM. AVG up to 1 mM did not decrease ethylene formation in either *Marsilea* or *Regnellidium* (Figure 2.2).

To address the question whether the inability of added ACC to increase ethylene production was a consequence of saturated ACC oxidase, α-aminoisobutyric acid (AIB), a competitive inhibitor of ACC oxidase (Satoh and Esashi, 1983), was applied. Concentrations of up to 5 mM AIB had no effect on ethylene production in *Marsilea* and in *Regnellidium*, but completely inhibited ethylene formation in *Arabidopsis* (Figure 2.3).

Effect of Iron.

Ethylene production in higher plants is dependent upon ferrous iron due to the requirement of ACC oxidase for it as a cofactor (Bouzayen et al., 1991). To test whether iron had any effect in *Regnellidium* and *Marsilea*, leaf discs were incubated with 1,10-phenanthroline, a chelator of ferrous iron. This treatment resulted in inhibition of ethylene production in *Arabidopsis*, *Marsilea*, and *Regnellidium*. This inhibition could be reversed by incubation of the leaves or leaf discs on 0.2 M $FeSO_4$ (Figure 2.4). Thus, despite the apparent absence of ACC oxidase in the ferns, iron is required for ethylene production.

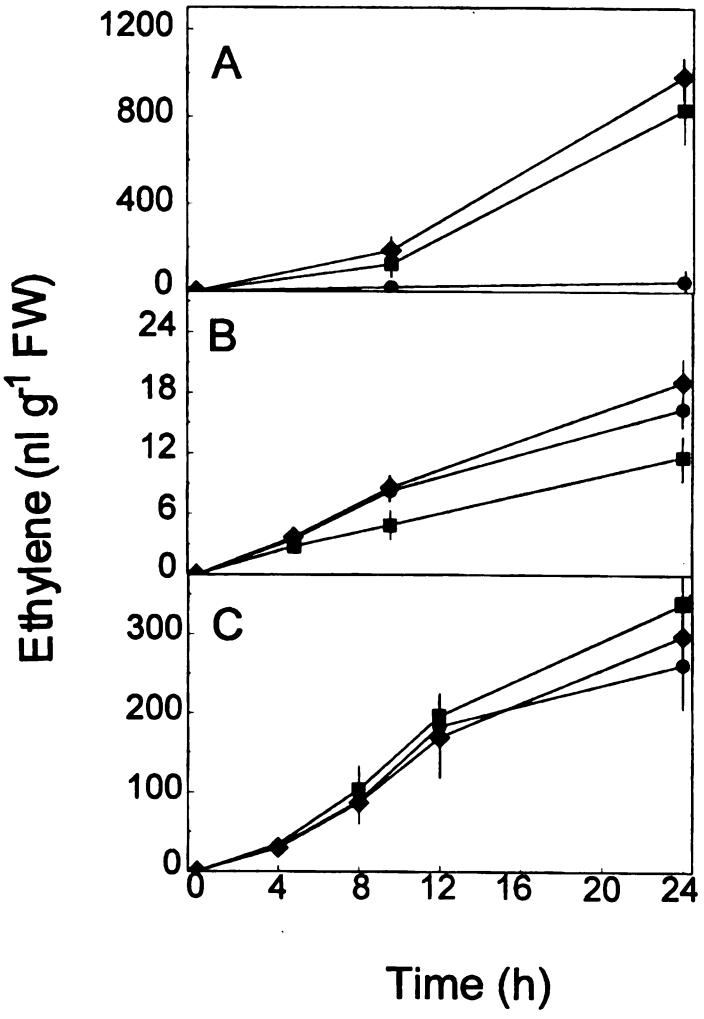

Figure 2.1A-C. Effect of 1-aminocyclopropane-1-carboxylic acid (ACC) on ethylene production in *Arabidopsis* (A), *Marsilea* (B), and *Regnellidium* (C). Data points are the means of 4 observations from a representative experiment, and the error bars represent SE. • , control; ■, 0.1 mM ACC; ♦, 1 mM ACC. This experiment was repeated four times with similar results.

Detection of ACC in ferns.

The presence of ACC was detected in leaflets of both *Regnellidium* and *Marsilea* (Table 2.1) and confirmed by thin-layer chromatography (Figure 2.5). Zones of the thin-layer chromatograms were scraped off and assayed. Those that yielded ethylene had R_f values corresponding to that of standard ACC (Figure 2.6).

Radiolabeling experiments.

To confirm the conversion of methionine to ACC and to check whether methionine was converted to ethylene, radiolabeling experiments were performed. Little [^{14}C]methionine was converted to [^{14}C]ethylene by either *Marsilea* or *Regnellidium* (Table 2.2; see also Cookson and Osborne, 1978). To assess whether the small conversion observed occurred via an ACC-dependent route, [^{14}C]methionine was applied together with [^{12}C]ACC to leaflet discs. The addition of [^{12}C]ACC should decrease incorporation of label into ethylene if the conversion occurs via ACC as an intermediate. This occurred in *Arabidopsis* but not in *Marsilea* and *Regnellidium* (Table 2.2). Further evidence in support of an ACC-independent pathway in ferns came from experiments where [^{14}C]methionine was fed to leaflet discs of *Regnellidium*, and the specific radioactivity of C-2 and C-3 of ACC was compared to that of ethylene. The specific radioactivity of the two carbon atoms of ACC that give rise to ethylene was close to 30-fold higher than the specific radioactivity of ethylene, indicating that the major part of ethylene was not derived from ACC (Table 2.3). In contrast, the specific radioactivities of C-2 and C-3 of ACC and of ethylene were approximately the same in *Arabidopsis*, as would be expected if ethylene were derived from ACC.

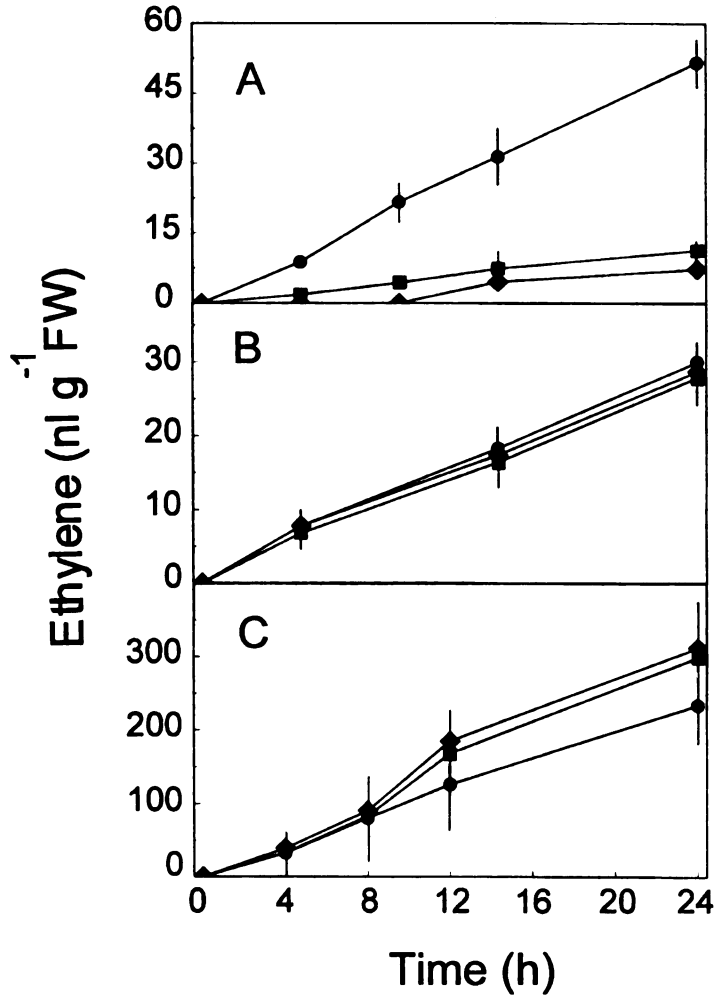

Figure 2.2A-C. Effect of aminoethoxyvinylglycine (AVG) on ethylene production in *Arabidopsis* (A), *Marsilea* (B), and *Regnellidium* (C). Data points are the means of 4 observations from a representative experiment, and the error bars represent SE. ●, control; ■, 0.1 mM AVG; ♦, 1 mM AVG. This experiment was repeated four times with similar results.

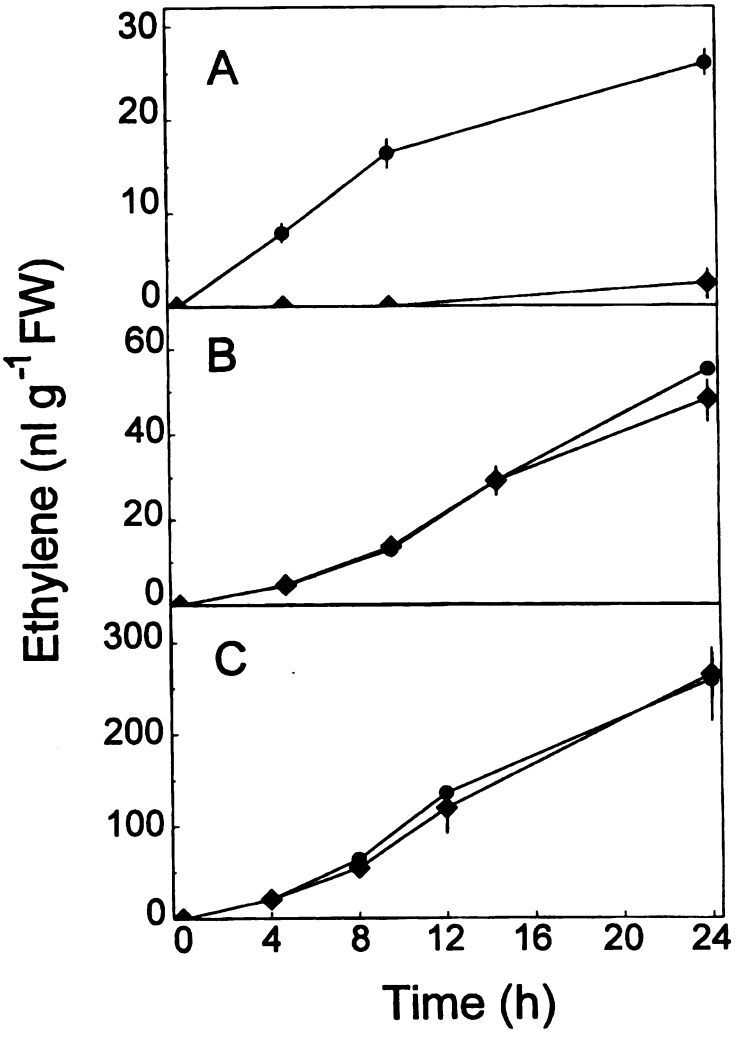

Figure 2.3A-C. Effect of α-aminoisobutyric acid (AIB) on ethylene production in *Arabidopsis* (A), *Marsilea* (B), and *Regnellidium* (C). Data points are the means of 4 observations from a representative experiment, and the error bars represent the SE. •, control; ♦, 5 mM AIB. This experiment was repeated four times with similar results.

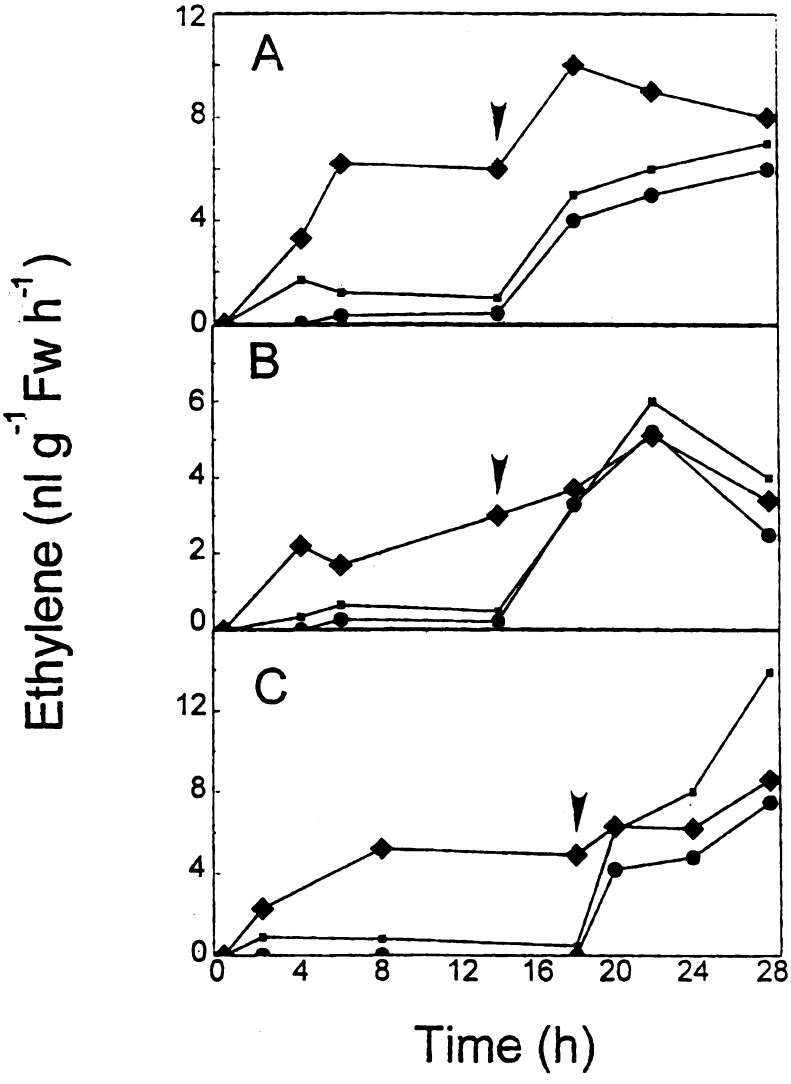

Figure 2.4. Ethylene production in the presence of the iron chelator 1,10-phenanthroline (PA) in *Arabidopsis* (A), *Marsilea* (B), and in *Regnellidium* (C). Transfer of the leaf discs or leaves to 0.2 M $FeSO_4$ reversed the inhibition. The position of the arrow indicates the time at which transfer occurred. The experiment is representative of two similar experiments. ♦, control; ■ , 0.2 mM PA; • , 2 mM PA.

Table 2.1. 1-aminocyclopropane-1-carboxylic acid (ACC) levels in *Arabidopsis, Marsilea* and *Regnellidium*. ACC was extracted from the tissue and measured by the method of Lizada and Yang (1979). The data represent the average of three experiments ± SE.

Sample	ACC levels (nmol g^{-1} FW)
Arabidopsis	0.28 ± 0.05
Marsilea	0.12 ± 0.09
Regnellidium	0.34 ± 0.11

To further test whether ACC was converted to ethylene in fern leaflets and to verify the results of Figure 1, radiolabeling studies were carried out using [^{14}C]ACC. Some conversion of [^{14}C]ACC to [^{14}C]ethylene occurred in both *Marsilea* and in *Regnellidium* but, compared to the conversion in *Arabidopsis*, it was low (Table 2.4).

Extracts of *Regnellidium* leaflets and *Arabidopsis* leaves that had been labeled with [^{14}C]methionine were chromatographed on thin-layer plates. The chromatogram was divided into ten zones, and the eluate of each zone was subjected to the ACC assay of Lizada and Yang (1979). The amount of ethylene produced from each zone, as well as the level of radioactivity associated with it, were determined (Figure 2.5). A region of the thin-layer chromatogram, which corresponded to the R_f of standard ACC, released ethylene in the Lizada-Yang assay and also exhibited a peak of radioactivity. When extracts of [^{14}C]ACC-labeled leaflets were chromatographed, no radioactive metabolites were detected (results not shown).

Presence of ACC Synthase.

Because ACC is present in both ferns, we tested whether ACC synthase activity was present as well. Low but significant levels of ACC synthase activity could

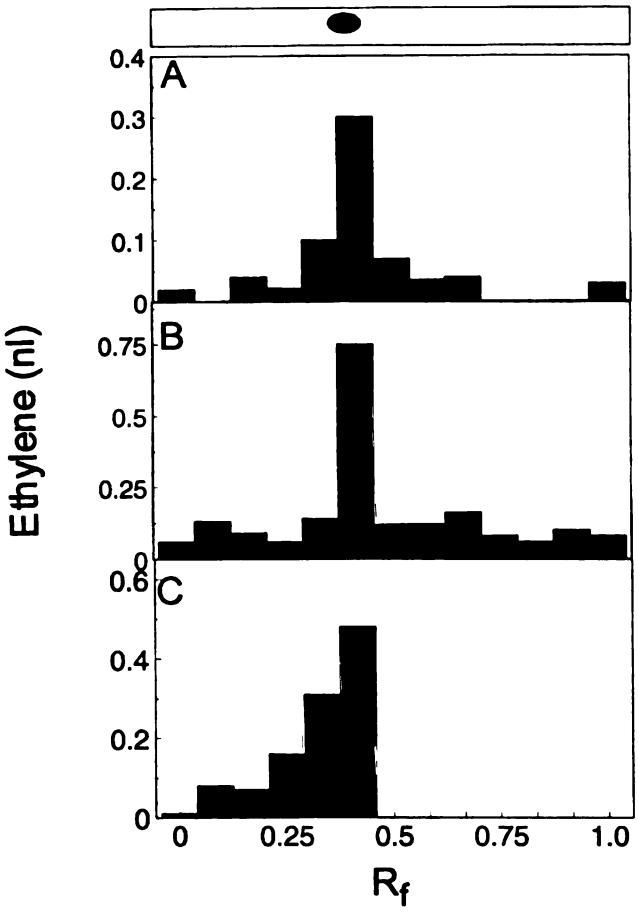

Figure 2.5A-C. Thin-layer chromatography of extracts from the leaflets of *Marsilea* (A), leaves of *Arabidopsis* (B), and leaflets of *Regnellidium* (C). After completion of the chromatographic run, the plates were divided into 1-cm zones which were scraped off and analyzed for ACC by the method of Lizada and Yang (1979). Ethylene evolved per 1-cm zone is plotted against the R_f. The region of the chromatogram which yielded the highest level of ethylene in the ACC assay corresponded to the R_f of ACC (0.45) run as a standard on the plates and stained with ninhydrin (illustrated in the bar above the charts). Similar results were obtained in three experiments.

Table 2.2. Incorporation of radiolabeled methionine into ethylene. Leaves from *Arabidopsis* and leaflet discs from *Regnellidium* and *Marsilea* were incubated on filter paper moistened with 74 kBq of [^{14}C]methionine in 1 ml of water. In a separate experiment, the leaves or leaflet discs were incubated with [^{14}C]methionine to which unlabeled ACC (100 μM) had been added. All experiments gave similar data.

Sample	Total C_2H_4 (nl h^{-1} g^{-1})	[^{14}C]C_2H_4 (Bq $nmol^{-1}$)
Arabidopsis[a]	9.4	396
Regnellidium[b]	9.3	86
Marsilea[c]	2.0	19
Arabidopsis[b] + ACC	64.3	86
Regnellidium[b] + ACC	9.2	73
Marsilea[c] + ACC	3.1	20

[a] Average of 4 experiments
[b] Average of 3 experiments
[c] Average of 2 experiments

consistently be detected in leaflets of *Marsilea* and *Regnellidium* (Table 2.5). The inhibition of this activity by the addition of 0.1 mM AVG to the *Regnellidium* extract confirmed its identity as ACC synthase.

Cloning of the gene for ACC Synthase from Marsilea.

ACC synthase has seven regions that are highly conserved among species (Kende, 1993). Two such regions were chosen for design of degenerate PCR primers. These primers were used in RT-PCR to amplify a fragment of the predicted size, around 320 bp. The sequence of the fragment shared a high degree of identity, at the amino acid level, with other ACC synthases. An alignment of the deduced amino acid sequence of the cloned *Marsilea* ACC synthase gene fragment with several other ACC synthases is shown in Figure 2.7. Northern blots of RNA from *Marsilea*, *Regnellidium*, pea, and *Arabidopsis* using the cloned fragment as a probe were tried but there was cross-reaction with *Arabidopsis* and pea. It would therefore seem that in order to draw conclusions about *Marsilea* ACC synthase gene expression, the full-length sequence would need to be

Table 2.3. Determination of the specific activity of ACC and ethylene produced by *Regnellidium* and *Arabidopsis*. Leaflets from *Regnellidium* and leaves from *Arabidopsis* were placed on filter paper saturated with 74 kBq [^{14}C]methionine in 25-ml Erlenmeyer flasks. After 4 h of incubation, the gas phase was transferred to a syringe, an aliquot of the gas phase was analysed for ethylene, and the ethylene was adsorbed to mercuric acetate. The leaves or leaflets were analyzed for ACC according to Lizada and Yang (1979). The ethylene produced during the ACC assay was transferred to a syringe, measured, and adsorbed to mercuric acetate. The radioactivity associated with the mercuric acetate was determined by scintillation counting. The data shown are from one experiment that was repeated with similar results.

Sample	[^{14}C]C_2H_4 (kBq nmol^{-1})	[^{14}C]ACC (kBq nmol^{-1})	Ratio [^{14}C]ACC / [^{14}C]C_2H_4
Arabidopsis	329	385	1.2
Regnellidium	41	1136	28

Table 2.4. Incorporation of radiolabeled ACC into ethylene. Leaves of *Arabidopsis* and leaflet discs from *Regnellidium* and *Marsilea* were placed on filter paper containing 37 kBq [^{14}C]ACC in 1 ml of water. The ethylene produced was adsorbed to mercuric acetate, and the radioactivity determined by scintillation counting. The data represent the average of three experiments.

Sample	Total C_2H_4 (nl h^{-1} g^{-1})	[^{14}C]C_2H_4 (kBq nmol^{-1})
Arabidopsis	17.7	2.5
Regnellidium	9.4	0.20
Marsilea	1.5	0.04

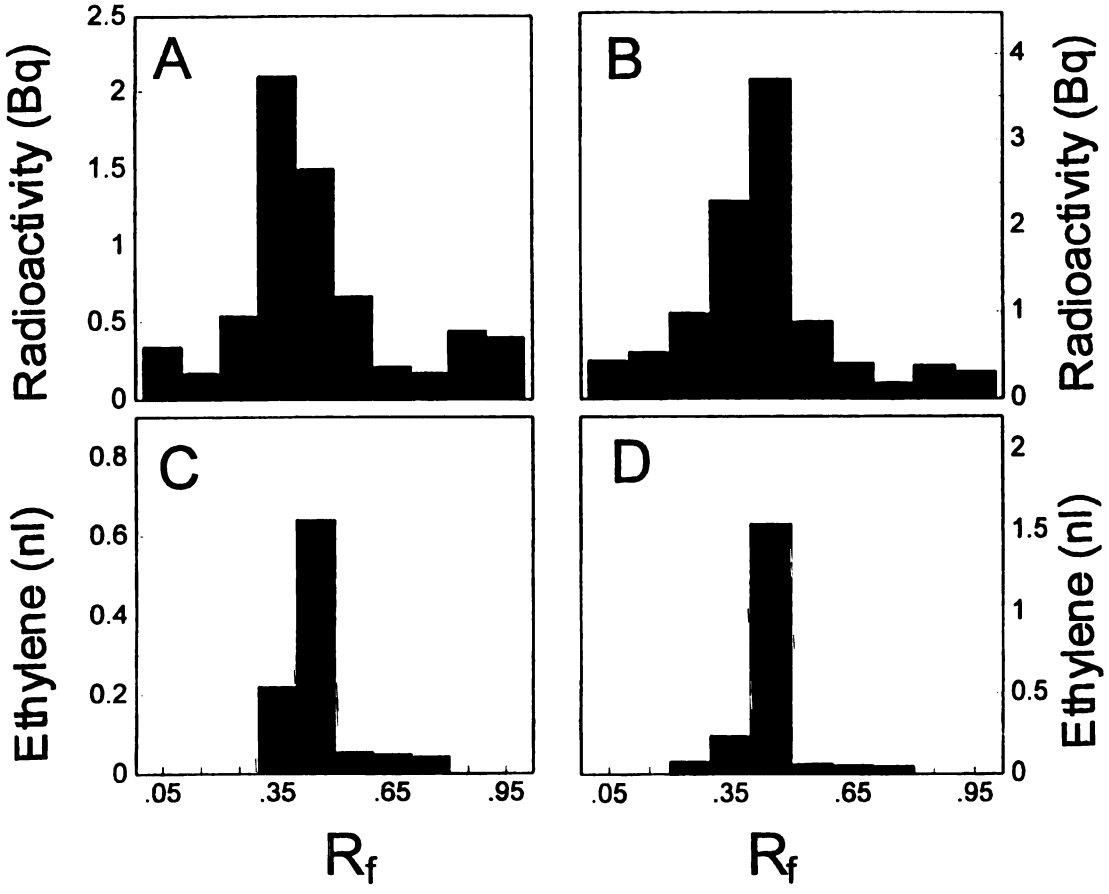

Figure 2.6A-C. Thin-layer chromatography of extracts from leaves of *Arabidopsis* (A,C) and leaflets of *Regnellidium* (B,D) which had been treated with [^{14}C]methionine. After completion of the chromatographic run, the plates were divided into 1.5-cm zones which were scraped off and analyzed for ACC by the method of Lizada and Yang (1979). The radioactivity associated with ethylene (A,B) and the amount of ethylene evolved per 1.5-cm zone (C,D) is plotted against the R_f. The region of the chromatogram which yielded the highest level of ethylene in the ACC assay corresponded to the R_f of standard ACC.

Table 2.5. ACC synthase activity in *Marsilea* and *Regnellidium* leaflets and in *Arabidopsis* leaves. The data represent the mean of three experiments ± SE.

Sample	C_2H_4 (pmol h^{-1} g^{-1} FW)
Marsilea	2.1 ± 0.5
Regnellidium	2.5 ± 0.6
Arabidopsis	3.1 ± 0.7
Regnellidium + heat	not detected
Regnellidium + 100 μm AVG	0.4 ± 0.2

obtained and gene-specific probes designed.

Effect of Submergence.

Previous studies have shown that *Regnellidium*, like rice, responds to submergence with petiole elongation. In rice, submergence results in increased ethylene synthesis. We tested whether the submergence of *Regnellidium* also results in increased ethylene synthesis. Submergence resulted in an increased growth rate; this increase in growth could be partially mimicked by application of ethylene (Figure 2.8). Leaf discs from plants that had been submerged had higher rates of ethylene evolution (Figure 2.9). To test whether this increased production resulted from synthesis or release due to accumulation following submergence, ethylene evolution of submerged leaflets was monitored at 25° and 4° C. Ethylene evolution decreased by 78% and 82% in submerged and control leaflets respectively, incubated at 4°C (Figure 2.9). As release of the entrapped ethylene would occur equally well at both 4°C and 25°C, these results indicate that at least part of the increased ethylene evolved from submerged leaflets is due to increased synthesis.

DISCUSSION

The results support the notion that at least the major part, if not all, of the ethylene synthesised by *Regnellidium* and *Marsilea* is derived from a precursor other than ACC, despite the fact that the initial step of the pathway, namely the formation of ACC from methionine, is functional in both ferns. This conclusion is based upon the following results obtained with *Marsilea* and *Regnellidium* leaflets: (1) ACC failed to stimulate ethylene formation, (2) AVG and AIB failed to inhibit ethylene formation, (3) ACC and ACC synthase were present, (4) [^{14}C]methionine was converted to [^{14}C]ACC but [^{14}C]methionine and [^{14}C]ACC were poorly converted into ethylene, and (5) the specific radioactivity of ACC formed from [^{14}C]methionine was much higher than the specific radioactivity of the ethylene evolved. In all of these experiments, leaves of *Arabidopsis* were used as positive controls and gave results that were consistent with the functioning of the ACC-dependent pathway. These results are consistent with those of Osborne et al. (1996).

Two pathways of ethylene biosynthesis have been described in microorganisms (for a review, see Fukuda et al., 1993). In *Escherichia coli*, ethylene is synthesized from methionine via 2-keto-4-methylthiobutyric acid (KMBA). The enzyme that catalyzes this conversion has been purified and has been shown to be a NADH:Fe^{3+} oxidoreductase. In the second pathway, present in *Penicillium digitatum* and in *Pseudomonas syringae*, ethylene is synthesised from α-ketoglutarate. The enzyme catalyzing this reaction has been purified, and the gene encoding it has been cloned. It is similar to higher-plant ACC oxidase in that it requires oxygen and Fe^{2+}, and is affected similarly by a number of inhibitors. The low level of conversion of [^{14}C]methionine to [^{14}C]ethylene in ferns

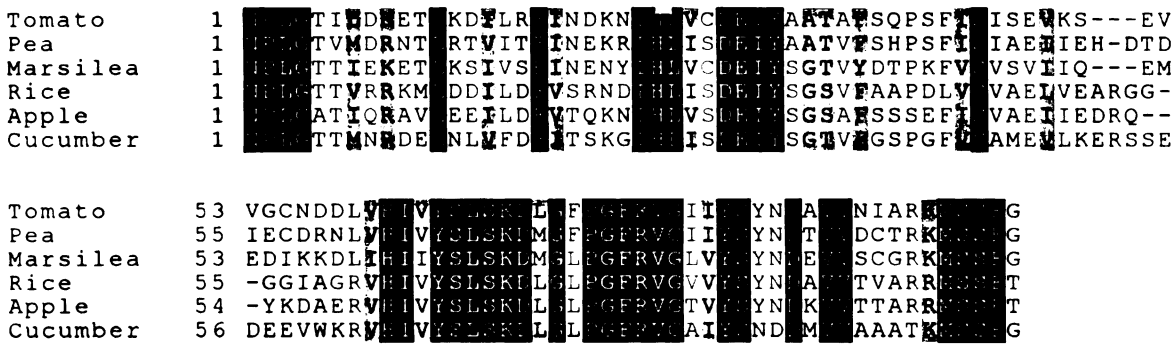

Figure 2.7. Alignment of the deduced amino acid sequence of the 300 bp RT-PCR product obtained from *Marsilea* with ACC synthases from pea (AF0164959), tomato (AB013100), rice (X97066), apple (U73816), and winter squash (U37774). The amino acid shaded in black are identical in all sequences. Grey areas indicate similar amino acids in all sequences.

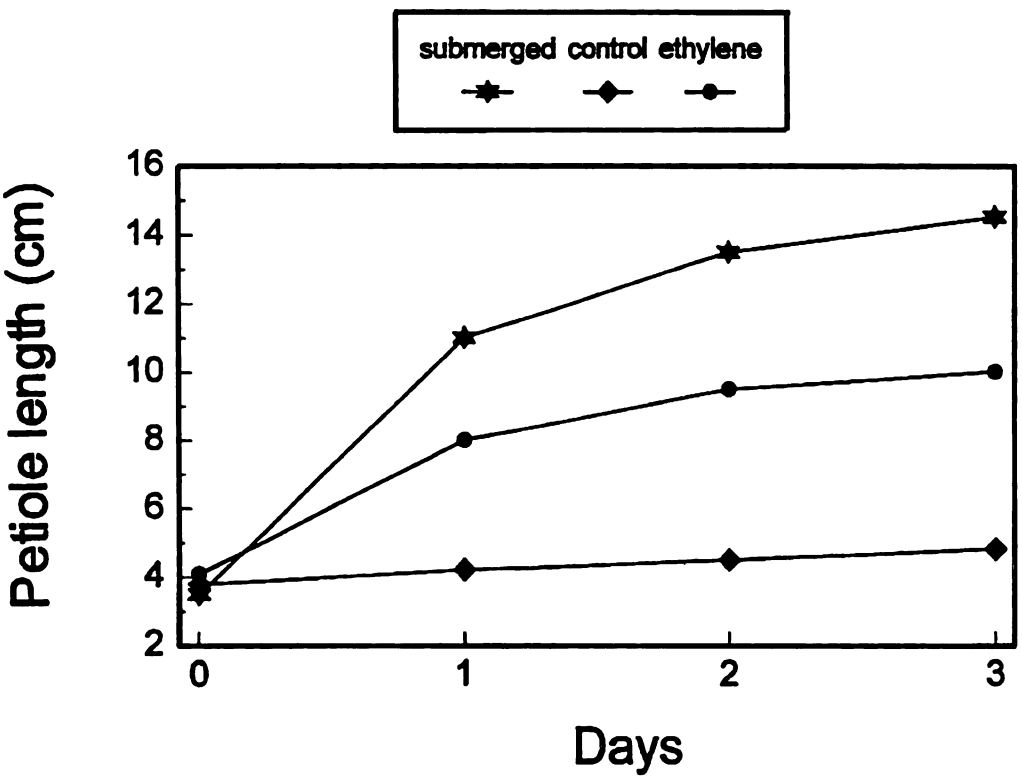

Figure 2.8. Submergence response in *Regnellidium*. *Regnellidium* plants were submerged such that the leaflets were held below the surface of the water. Ethylene treatment was carried out as described in Materials and Methods. The data shown here are representative of three experiments.

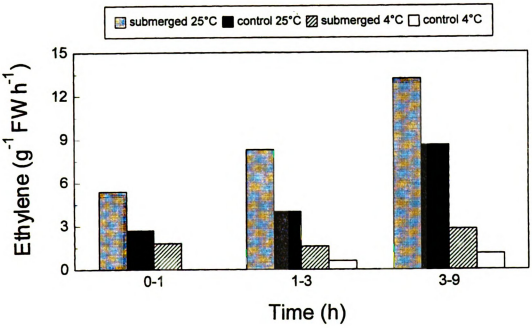

Figure 2.9. Ethylene evolution at 25° and 4° C from control (non-submerged) *Regnellidium* leaflets and from *Regnellidium* leaflets that had been submerged for three days. The data shown here are representative of two experiments.

(Table 2.2; Cookson and Osborne, 1978) is evidence against the existence of the KMBA-dependent pathway in ferns. The second pathway does not seem to operate in ferns either as [U-^{14}C]glutamate added to leaflets was not converted to ethylene (results not shown). However, the pathway that does exist in ferns is similar to that in higher plants and bacteria in having a requirement for iron.

A number of other ACC-independent pathways of ethylene synthesis have been reported. Copper-induced ethylene biosynthesis in *Spirodela* may be mediated by peroxidation of unsaturated fatty acids, e.g., linoleic acid (Mattoo et al., 1992). An ACC-independent pathway is also indicated in lichens (Lurie and Garty, 1991), sweet potato infected by *Ceratocystis fimbriata* (Hyodo and Uritani, 1984), stressed pine needles (Chen and Wellburn, 1989), and mung bean epidermal cells (Todaka and Imaseki, 1985). Because of its simple chemical structure, there are many compounds that may be converted to ethylene through various chemical reactions. This makes identification of the precursor to ethylene difficult.

The evolution of land plants required a series of changes in vascular development, and potentially in biochemical pathways. It is possible that one such change is the adjustment to differing water conditions and different gas concentrations, including oxygen. The acquisition of an ethylene biosynthetic pathway that was more highly regulated may be an event that is related to the transition from water to land. Semi-aquatic ferns may possess a means of sensing of the low oxygen concentrations that would be present when a plant is submerged; and this sensing may involve ethylene production but is not necessarily responsive to the same stimuli that would exist for a land plant. *Regnellidium* has a similar adaptation response to submergence as higher

plants, but appears to have a different pathway for synthesis of the hormone that is involved in this response. Another variation in the ethylene biosynthetic pathway in an aquatic plant is that of *Potamogeton pectinatus* which responds to submergence by stem elongation and contains ACC but does not produce ethylene (Summers et al., 1996). The submergence effect provides a useful physiological condition in which increased ethylene synthesis can be related to changes in ACC levels, ACC synthase activity, and potentially be useful in the purification of enzymes of the fern pathway.

The presence of ACC synthase and the apparent absence or very low *in vivo* activity of ACC oxidase in *Regnellidium* and *Marsilea* is interesting from an evolutionary standpoint. Because ACC synthases and aminotransferases are related and because phylogenetic analysis indicates that the polymorphism of ACC synthase genes arose prior to the divergence of monocots and dicots (Zarembinski and Theologis, 1994), it will be interesting to compare the ACC synthase gene(s) and proteins(s) of *Regnellidium* and *Marsilea* to those of higher plants. This is difficult to do only from the partial sequence of *Marsilea* ACC synthase that is from a region conserved in all ACC synthases. Obtainment of the full-length clone should be useful in this regard. Similarly, it will be interesting to compare the ethylene-forming enzyme(s) of these two ferns to those of fungi and bacteria and to ACC oxidase of higher plants.

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Chapter 3

Characterization of the 9-*cis*-epoxycarotenoid dioxygenase gene family and the regulation of ABA biosynthesis in avocado

ABSTRACT

Avocado (*Persea americana*) is a climacteric fruit that exhibits a rise in ethylene as the fruit ripens. This rise in ethylene is accompanied by a series of molecular and biochemical changes that culminate in a soft, flavorful fruit. One such change is a rise in abscisic acid (ABA), with the highest level occurring just after the peak in ethylene production. ABA is synthesized from carotenoid precursors, which in leaves are present in large quantities in comparison with ABA, and thus are not limiting for ABA synthesis. The cleavage reaction produces xanthoxin, which can subsequently be converted into ABA via ABA-aldehyde. Much evidence supports the cleavage reaction as the regulatory step in ABA synthesis. This cleavage reaction is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED), the gene for which was first cloned from the *vp14* viviparous mutant of maize. Three genes encoding 9-*cis*-epoxycarotenoid dioxygenase cleavage-like enzymes were cloned from avocado fruit. Two genes, *PaNCED1* and *PaNCED3*, are strongly induced as the fruit ripens, and share approximately 60% identity at the amino acid level with the maize gene, *Vp14*. The other gene, *PaNCED2*, is constitutively expressed during fruit ripening and lacks a chloroplast signal peptide. It is, therefore, unlikely to be involved in ABA biosynthesis. All three genes were heterologously expressed, and the recombinant protein used to test for enzymatic function. Recombinant PaNCED1 and PaNCED3 were capable of *in vitro* cleavage of 9-*cis*-xanthophylls into xanthoxin and C_{25} epoxycarotenoids. Taken together, the results indicate that ABA

biosynthesis in fruit is regulated at the level of carotenoid cleavage.

INTRODUCTION

Fruit ripening involves a complex series of biochemical events in which the tissue undergoes programmed changes in texture, aroma, coloration, flavor, and firmness (Brady, 1987). Climacteric species, such as avocado (*Persea americana*), are characterized by the autocatalytic production of the ripening hormone ethylene and a ripening-related transient burst in CO_2 evolution (Biale and Young, 1981). In avocado, the increase in ethylene production is followed by an increase in abscisic acid levels (ABA) (Adato et al., 1976). While ethylene induces the synthesis of many genes involved in fruit ripening (Brady, 1987), it is not known whether the rise in ethylene is related to the increase in ABA in avocado. Further, the role that ABA plays in the ripening process is also unknown. Ripening avocado fruit produces high levels of ABA and thus provides an ideal system in which to study the regulation of ABA biosynthesis.

ABA plays a role in adaptation to various stresses (e.g. cold and osmotic stress), and also during developmental changes, such as seed germination and embryo development (Zeevaart and Creelman, 1988). The increase in ABA levels in water-stressed leaves can be prevented by both transcriptional inhibitors (Guerrero and Mullet, 1986), and translational inhibitors (Stewart et al., 1986), indicating that RNA and protein synthesis are necessary to mediate the drought-induced increase in ABA levels.

ABA is synthesized from carotenoid precursors that are present in relatively large quantities in most photosynthetic tissues in comparison to ABA (Norman et al., 1990). Biochemical (Zeevaart and Creelman, 1988), and genetic data (Rock and Zeevaart, 1991) have indicated that the cleavage of 9-*cis*-xanthophylls is likely the key regulatory step in

the ABA biosynthetic pathway. The cleavage of 9-*cis*-xanthophylls produces a C_{25} apocarotenoid and xanthoxin (Zeevaart, 1999). The xanthoxin can be subsequently converted into ABA via ABA-aldehyde. The enzymes that carry out these later conversions (xanthoxin into ABA-aldehyde and ABA-aldehyde into ABA) are constitutively expressed in leaves (Sindhu and Walton, 1988) and are therefore not limiting for ABA biosynthesis. Other steps in the ABA biosynthetic pathway, such as the conversion of zeaxanthin into violaxanthin catalyzed by zeaxanthin epoxidase, show little up-regulation during water stress of leaves (Burbidge et al., 1997a). This further supports the notion that another part of the ABA biosynthetic pathway must be regulatory.

Confirmation of the regulatory nature of the cleavage reaction was provided by the cloning of *Vp14*. *vp14* mutants of maize are viviparous and exhibit a defect in ABA biosynthesis (Tan et al., 1997). The derived protein sequence of *Vp14* is related to lignostilbene dioxygenases, bacterial enzymes that catalyze a double bond cleavage reaction analogous to the carotenoid cleavage reaction in ABA biosynthesis. Recombinant VP14 protein catalyzes the cleavage of 9-*cis*-xanthophylls into ABA (Schwartz et al., 1997) in a reaction that requires oxygen, ferrous iron, ascorbate, and a detergent for activity *in vitro*. Northern analysis of maize leaves showed that *Vp14* is induced during wilting in parallel with the increase in ABA levels (Tan et al., 1997).

In the time since the cloning of *Vp14*, a number of genes with sequence similarity to *Vp14* have been reported (Watillon et al., 1998; Neill et al., 1998; Burbidge et al., 1997b), and a number of additional homologous genes are present in the database. Based upon the degree of sequence similarity of these genes with *Vp14*, it can be inferred that the encoded proteins catalyze reactions in which a double bond is oxidatively cleaved,

yielding two products with aldehyde groups at the site of cleavage. While not all of the homologous genes are necessarily involved in ABA biosynthesis, it would seem as though at least some are. In particular, the *notabilis* mutant of tomato is impaired in the ability to convert C_{40} precursors to xanthoxin, and hence *notabilis* mutants have a reduced ABA content (Parry et al., 1988) and exhibit a wilty phenotype. The cloned gene is highly homologous to *Vp14* (Burbidge et al., 1999), and message levels of this gene are increased during leaf wilting. The nomenclature now used for designating genes that have homology to *Vp14* is nine-*cis*-epoxycarotenoid dioxygenase genes or *NCED*.

The regulation of ABA levels in fruit has not previously been investigated. Labeling studies of ABA using $^{18}O_2$ have shown that the indirect pathway (i.e. synthesis from C_{40} carotenoids) of ABA biosynthesis in leaves is operational in both avocado and apple fruit (Zeevaart et al., 1989). Carotenoid levels in various fruits appear to be high enough so as not to be limiting for ABA biosynthesis, and therefore the cleavage of xanthophylls is probably the regulatory reaction in fruit. To test this, three *Vp14* homologs were cloned from ripening avocado fruit, and their expression during the ripening process was monitored. Two of these genes (*PaNCED1* and *PaNCED3*) are induced in parallel as the fruit ripens. A third gene, *PaNCED2* exhibits constant expression both during fruit ripening and during the wilting of leaves, suggesting that it has a housekeeping role. The tissue-specific differences in expression of the NCED genes, and differences in the activities of the expressed proteins, may have implications for their *in vivo* physiological role in regulating ABA biosynthesis.

MATERIALS AND METHODS

Plant Material.

Avocado fruit (*Persea americana,* cv. Lula) were kept at room temperature for up to 14 days in a tray moistened with wet paper towels and covered with Saran wrap to prevent dessication. Each day individual fruits were incubated in sealed containers, and after a period of time, 1 ml of the gas phase was removed for ethylene determination by gas chromatography. On various days during the ripening period, one avocado fruit was harvested by removing the rind and cutting it into small pieces that were then frozen in liquid N_2 and stored at -80 °C to be used for subsequent analysis.

Avocado seedlings were grown from seeds of Lula under greenhouse conditions. Mature leaves from the top of the plant were wilted to differing percentages of their fresh weight, up to a maximum 80%, using a pressure chamber (Boyer, 1995). After removal from the chamber, the leaves were left in a moist plastic bag in the dark for 4 h, then frozen in liquid N_2.

Pea seeds (*Pisum sativum* var. Little Marvel) were supplied by Olds Seed Company (Madison, WI). Seedlings used for isolation of chloroplasts were grown at 25°C in a growth chamber for 9 to 12 days.

Extraction and Purification of ABA and Carotenoids.

ABA was extracted with acetone and purified as described by Zeevaart et al. (1989). A small amount of [^{3}H]ABA was added to each sample to determine the percentage recovery. Quantification was by GC with electron capture detection (ECD) using endrin as an internal standard.

Extraction and analysis of carotenoids were performed according to Rock and

Zeevaart (1991). Carotenoids were quantified by integration of the peak area for absorbance at 436 nm with a Waters 740 data module. A C_{25}-apocarotenoid, *trans*- β-apo-8'-carotenal (Fluka), was added to each sample as a standard so that percentage recovery could be calculated. Corrections were made for differences in extinction coefficient and for the differences in absorption at 436 nm and the maximal absorption for each carotenoid.

RNA Extraction and Northern Analysis.

Total RNA was extracted from avocado mesocarp using a phenol-chloroform method (Vanlerberghe et al., 1992). Leaf RNA was isolated by a method that is a combination of methods by Callahan et al. (1989) and Ainsworth (1994). Tissue was ground in liquid nitrogen to a fine powder using a mortar and pestle. The ground tissue was transferred to a tube containing extraction buffer [100 mM sodium acetate, 500 mM NaCl, 50 mM Na_2EDTA, 1.4% sodium dodecyl sulphate, 2% polyvinylpyrrolidone (M_r 40,000), 1% β-mercaptoethanol] and the homogenate was placed at 65°C for 30 min. Cellular debris was removed by centrifugation (10,000g, 10 min), and the supernatant was extracted with distilled phenol. After centrifugation (10,000g, 10 min), the aqueous phase was extracted with phenol/chloroform and re-centrifuged. The aqueous phase was extracted with chloroform and centrifuged (10,000g, 10 min). The aqueous phase was placed on ice, and 0.1 vol of 3 M Na-acetate (pH 4.8) was added. The pH was brought to 5 by the addition of glacial acetic acid. After 2 h on ice, the RNA was pelleted, and washed once with 80% cold ethanol. The pellet was resuspended in 2 ml of distilled water and precipitated overnight with 1/4 volume of 10 M LiCl. The RNA was pelleted by centrifugation (10,000g, 10 min), and washed with 80% cold ethanol. The pellet was

resupended in ddH_2O and RNA quantitated by absorbance at 260 and 280 nm determined. Northern analysis was carried out by electrophoresis of 30 μg of total RNA on 1.2 % (w/v) agarose gels containing 2.2% (v/v) formaldehyde according to Maniatis et al. (1982) and transferred onto nylon membranes (Hybond N+, Amersham). Hybridization was performed at 65°C using Church-Gilbert hybridization buffer (Church and Gilbert, 1984). Blots were washed first at low stringency (2x SSC, 0.1% SDS, room temperature - 2x, 15 min each followed by 15 min, 2x at 65°C), then at high stringency (additional wash 0.2x SSC, 0.1% SDS, 30 min at 65°C). Following hybridization and development of the autoradiograms, blots were stripped in 0.2% hot SDS and re-probed.

Genomic Southern Analysis.

Avocado (Lula) genomic DNA was isolated from leaves using an extraction method based on the detergent cetylmethylammoniumbromide (Hulbert and Bennetzen, 1991). DNA digestion, gel and electrophoresis conditions were as described by Maniatis et al. (1982). Low stringency and high stringency wash conditions were performed as for Northern analysis.

Probe Synthesis and Labeling.

Plasmids (pGEMTeasy) containing the full-length *PaNCED* genes as inserts were digested with either *Not*I (*PaNCED3* and *PaNCED1*) or *Eco*R1 (*PaNCED2*), electrophoresized on agarose gels, and inserts were purified using a Qiagen gel extraction kit. Purified probes were labeled by random-prime labeling (Gibco-BRL) and non-incorporated nucleotides removed by spin chromatography. For gene-specific probes, JZ205 and JZ225 (see Table 3.1 for a list of primers) were used to amplify a fragment corresponding to the 3' untranslated region of *PaNCED3*. The region amplified using

Table 3.1. Primers used in the amplification of 9-*cis*-epoxycarotenoid dioxygenase genes from avocado fruit.

Primer	Sequence	Gene Amplified	Orientation
JZ101	TTY GAY GGN GAY GGN ATG G	*NCED1*	S
JZ117	GCY TTC CAN AGR TCR AAR CA	*NCED1*	AS
JZ108	ACR TAN CCR TCR TCY TC	*NCED2*	AS
JZ110	ATG ATN CAY GAY TTY GC	*NCED2*	S
JZ120	GAG GCG GCA AGT GAT	*NCED2*	AS
JZ121	CAG TTA TTG CGA AAT CAT GC	*NCED2*	AS
JZ147	AAA TGA GCT GTA TGA AAT GA	*NCED2*	S
JZ148	CAG TGG AAT CGT GAA AGA GAA	*NCED2*	S
JZ153	AAC GAA TCT GCG CAG GCA TTT CCG	*NCED2*	S
JZ161	AAC TTG AAA GCA GTA	*NCED2*	S
JZ162	GTT TAC GTA GTT TTA TTT TGC TCG	*NCED2*	AS
JZ184	ACG CCG GTT CCG CTG GAG CCG TCG	*NCED1*	AS
JZ185	ACA TCG CGA GGA GGT GCC GGT TGA A	*NCED1*	AS
JZ186	GAT TCA TGA CTT CGT CAT TAC TGA	*NCED1*	S
JZ187	TTC GTC ATA ATT CCA GAC CAG CAG	*NCED1*	S
JZ200	CCA ACA ACC CAT TGC TCT TCT	*NCED1*	S
JZ205	ATT ATA GAG AAC CAG CTA AGG TAC	*NCED3*	AS
JZ206	TTC CAC ACC TAA AAC AAA CAA ATT	*NCED3*	AS
JZ222	CCC GGG GAC GCA AGC CTA AT	*NCED1*	AS
JZ223	TCG ACT CAC TGA GTC GCA	*NCED1*	S
JZ224	TAC AAG CAG TGG AAG AAG GGA AGG	*NCED1*	AS
JZ225	GAA CAA GAC CCT GAG ACT GAG	*NCED3*	S
JZ245	GTC CAA GGC GAA GGC CAG CAG TCC	*NCED3*	AS
JZ240	CAT TCC CTC GTC GGC GTT CAC CA	*NCED3*	AS
JZ250	ACT CTT ATG TCA ATG GCT ACT CCT	*NCED3*	S

Y=C/T, N=A/G/C/T, R=A/G

S= sense, AS=antisense

these primers extended from position 1939 to 2239 bp, resulting in a PCR product of 300 bp. Gene-specific primers JZ223 and JZ224 (corresponding to position 1827 and position 2162 bp, respectively) were used to amplify a 335-bp gene-specific fragment from *PaNCED1*. For *PaNCED2*, a 200-bp gene-specific fragment was amplified using primers JZ161 (position 1742 bp) and JZ162 (position 1942 bp).

RT-PCR.

RNA was extracted from fruit as described above. The first strand cDNAs were synthesized by RT from 4 μg total RNA isolated from avocado fruit ripened for 8 days using MMLV reverse transcriptase (Gibco-BRL) and an oligo dT primer. These cDNAs were used as templates for RT-PCR using degenerate primers JZ101 and JZ117 for the amplification of *PaNCED1*, and degenerate primers JZ108 and JZ110 for the amplification of *PaNCED2.* These primers were designed based on the conserved regions of the tomato *NCED* and maize *Vp14* genes. Conditions for RT were as follows: 65°C, 5 min, followed by 45°C for 1 hr, followed by 75°C, 5 min. PCR amplification was performed as follows: 30 cycles of 94°C 1 min, 55°C 1.5 min, 72°C 1 min.

Amplification of Full-Length cDNAs by RACE -PCR.

To obtain the full-length nucleotide sequences for *PaNCED2* and *PaNCED1*, RACE-PCR was performed using a kit according to the manufacturer's instructions (Gibco-BRL). The 5' ends of each of the genes were amplified using the following gene-specific primers: JZ184 (nested) and JZ185 (outer) for *PaNCED1*; JZ121 (outer) and JZ120 (nested) for *PaNCED2*; JZ243 and JZ240 for *PaNCED3*. To amplify the 3' ends, the following gene-specific primers were used: JZ186 (outer) and JZ185 (inner) for *PaNCED1*; JZ148 (outer) and JZ147 (inner) for *PaNCED2.* Plasmids resulting from

cloning of the 3' end of Pa*NCED1* were heterogeneous as judged by the hybridization signal strengths on Southern blots probed with PCR fragment JZ101/JZ117 of *PaNCED1*. Sequencing of these plasmids revealed them to be a portion of a new gene that was related to PaNCED1. The new gene was designated *PaNCED3*, and 5' RACE (using JZ240 and JZ245) was used to obtain the full-length clone. Primers JZ200 (at the 5' end) and JZ222 (at the 3' end) were used in end-to-end PCR to obtain the full-length *PaNCED1*. Primers JZ162 (at the 3' end) and JZ153 (at the 5' end) were used in end-to-end PCR to obtain the full-length *PaNCED2*. Primers JZ 206 (at the 3' end) and JZ250 (at the 5' end) were used to obtain the full-length *PaNCED3*.

Cloning and DNA Sequencing.

The PCR products corresponding to either partial fragments or the full-length genes were ligated into pGEMTeasy (Promega) and then introduced into *Escherichia coli* DH5α. Plasmids were sequenced using a DNA sequencer with either the -21M13 or M13 sequencing primers.

Protein Expression and Purification.

PaNCED1 in pGEMTeasy was digested with *Not*I to clone into the *Not*I site of pGEX5-2 (Pharmacia). *PaNCED3* was excised from pGEMTeasy using *Not*I and cloned into pGEX5-1. *PaNCED2* was cloned into the *Eco*R1 site of pGEX5-2. The plasmids were transformed into BL21 cells. Overnight 4 ml cultures were inoculated into 200 ml of 2YT medium and grown at 37°C until the optical density reached 0.7. At that time, 200 mM IPTG was added, and the cultures transferred to a shaker at 25°C in the case of *PaNCED2* and *PaNCED3* or to 18°C in the case of *PaNCED1*. After 5 h for *PaNCED2* and-*3* , or 16 h for *PaNCED1*, cells were harvested by centrifugation at 12,000g for 10

min, washed once with Tris-buffered saline (TBS), pH 7, centrifuged at 12,000g for 10 min, and resuspended in 10 ml TBS. One ml of 100 mg/ml lysozyme was added and the suspension was left on ice 30 min before being frozen overnight at -20°C. The extract was thawed the next morning, 0.1 M DTT was added, and it was sonicated using a probe sonicator (Sonifier 450, Branson) in 15 sec pulses for approximately 6 min total. The extract was separated into soluble and insoluble fractions. The soluble fraction was added to a 1 ml 50% slurry of Glutathione Sephadex (Pharmacia), and incubated 2 h at 8°C. At this time the mixture was centrifuged in a table top centrifuge, the beads washed 3x with 1x TBS, and once with Factor Xa buffer. One-half ml of Factor Xa buffer and 25 units of Factor Xa (Pharmacia) were added, the beads were shaken for 3 h at room temperature. At this time 5 µl of 20% Triton X-100 were added, and incubation was continued for another hour. The eluted protein was collected and frozen at -80°C.

Assay of Enzymatic Activity of NCED.

Assay of the enzymatic activity of PaNCED1, 2, and 3 was performed as described by Schwartz et al. (1997). The cleavage reaction products were analyzed by HPLC on a µPorasil column (Waters) equilibrated with 90% (v/v) hexane and 10% ethyl acetate. The column was eluted with a linear gradient to 20% hexane and 80% ethyl acetate over 17 min. The xanthoxin and C_{25} products from the cleavage of 9'-*cis*-neoxanthin and 9-*cis*-violaxanthin were collected and identified by mass spectrometry according to Schwartz et al. (1997). A standard curve of xanthoxin was constructed by injecting known quantities and integrating the peak areas.

Binding and Chloroplast Import Assays.

Intact pea chloroplasts were isolated from 8- to 12-day-old pea seedlings and purified over a Percoll gradient as previously described (Bruce et al., 1994). Intact pea chloroplasts were reisolated and resuspended in import buffer (330 mM sorbitol, 50 mM Hepes/KOH, pH 8.0) at a concentration of 1 mg chlorophyll/ml.

The plasmid containing the gene for the small subunit of ribulose bisphosphate carboxylase/oxygenase (prSS) (Olsen and Keegstra, 1992) was linearized with *Pst*I and transcribed with SP6 polymerase. The plasmid containing outer envelope protein OM14 (Li et al., 1991) was linearized and transcribed using SP6. Plasmids containing the coding region for *PaNCED1*, *PaNCED2,* and *PaNCED3* were linearized and transcribed with T7 RNA polymerase. *PaNCED1*, *PaNCED2*, and *PaNCED3* were translated using a nuclease-treated rabbit reticulocyte system using the suggested protocol of the manufacturer (Promega). Likewise, the control plasmids pSS and OM14 were translated using a nuclease-treated rabbit reticulocyte lysate system. All proteins were radiolabeled using [^{35}S]-methionine.

Binding and import reactions were adapted from Young et al. (1999). Briefly, chloroplasts were pretreated with 6 μM nigericin for 10 min in the dark to deplete internal ATP levels. The precursor proteins [^{35}S]-PaNCED1, [^{35}S]-PaNCED2 and [^{35}S]-PaNCED3 were purified by gel-filtration according to the method of Olsen et al. (1989). All reactions contained intact chloroplasts corresponding to 25 μg chlorophyll in a final volume of 150 μl and 500,000 dpm of either [^{35}S]-prSS, [^{35}S]-OM14, [^{35}S]-PaNCED1, [^{35}S]-PaNCED2 or [^{35}S]-PaNCED3. Either no ATP, 0.1 mM ATP for binding, or 1 mM ATP for translocation was added to each of the reactions. All reactions were incubated

for 30 min at room temperature. Intact chloroplasts were recovered by sedimentation onto a 40% (v/v) Percoll cushion. The pellets were resuspended in lysis buffer (25 mM Hepes-KOH, pH 8.0/4 mM $MgCl_2$), and incubated on ice for 15 min. After ultracentifugation at 100,000g a total membrane and soluble fraction were obtained and solubilized in 2x SDS-PAGE sample buffer. All fractions were analyzed by SDS-PAGE (Laemmli, 1970), and autoradiography. Treatment of reactions with thermolysin was performed as described by Cline et al. (1984). For extraction of total envelope membranes, a large-scale import reaction was performed as described above, and fractionated using the method of Perry et al. (1994). Total envelope membranes were extracted with either high salt, sodium carbonate, or Triton-X100 according to the method of Tranel et al. (1995).

RESULTS

Cloning of NCED genes.

A number of conserved regions are present in 9-*cis*-epoxycarotenoid dioxygenase genes (Burbidge et al. 1997b). Degenerate primers JZ101 and JZ117 were used to amplify an approximate 1.1-kb fragment from day 8 cDNA. This new gene was designated *PaNCED1*. The full-length gene, obtained using RACE-PCR, contained an open reading frame of 1710 bp, with a 3' untranslated region of 377 base pairs, and a 5' untranslated region of 116 bp. The complete nucleotide sequence of PaNCED1 is presented in Figure 3.1. The predicted molecular weight of the protein is 63.1 kDa, slightly smaller than the predicted molecular weight of VP14. At the amino acid level, PaNCED1 was approximately 60% identical to VP14.

Degenerate primers JZ108 and JZ110 (see Table 3.1) were used to amplify an

approximately 600 bp fragment from cDNA of day 8 avocado fruit. This gene was designated *PaNCED2*. The full-length cDNA, obtained using 5' and 3' RACE, contained an open reading frame of 1575 bp encoding a protein with a predicted molecular weight of 59.6 kDa. The 3' and 5' untranslated regions were 226 and 166 bp, respectively. The nucleotide sequence of *PaNCED2*, along with the position of the start and stop codons, and the primers used in the amplification reactions, is presented in Figure 3.1. In comparison to VP14 and the tomato homolog, the deduced amino acid sequence was truncated at the amino-terminus and thus appeared to lack a transit peptide for chloroplast targeting. Overall, the gene shared approximately 30% identity at the deduced amino acid level with VP14, LeNCED1, and PaNCED1.

During each of the RACE procedures, Southern blotting of the minipreps corresponding to the 3' and 5' ends of the gene was performed to ensure that the newly amplified region cross-hybridized with the previously cloned portion. During the cloning of the 3' end of *PaNCED1*, it was noticed that the minipreps differed in terms of the strength of the hybridization signal when the fragment corresponding to JZ101/JZ117 was used as probe on Southern blots. These plasmids corresponding to the weaker signal on Southern blots were sequenced, and discovered to be a unique *NCED* gene. This gene, designated *PaNCED3*, was 60% identical at the amino acid level to *Vp14* and *LeNCED*, and 67% identical to *PaNCED1*. *PaNCED3* contained an open reading frame of 1878 bp, with 3' and 5' untranslated regions of 388 and 44 bp, respectively. The nucleotide sequence of *PaNCED3* is shown in Figure 3.1. An alignment of the deduced protein sequences of the three avocado genes with Vp14 is shown in Figure 3.2. Some properties of the three avocado genes are summarized in Table 3.2.

Figure 3.1. Nucleotide sequences of the three avocado *NCED* genes. The start and stop codons are indicated in bold and are underlined. The positions of the gene specific primers used are indicated with arrows above (for sense primers), or below (for antisense primers) the nucleotide sequences.

Nucleotide Sequence of *PaNCED1*

```
1     CACTGTAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGG
                              JZ200
53    CCGCGGGAATTCGATTCCAACAACCCATTGCTCTTCTCCCTCGTCATTGGAT

105   CTCAGCTGTCCAATGACAACCATCAGACAAAAACCCAAAACCTTCACAATCC

157   ACAGCTCTTTGCATTCCTCTCCTGTTCTTCACCTCCCCAAACTCCTCACTAC

209   TACTACTACTCCTCTTCATGAGAAGAGCCAAAGAGAATTGGGCTTGATCTTG

261   CAAGAGCCAAATAGGGCCAAGTGGAATTTTTTCCAAAGGGCTGCAGCTGTGG

313   CCTTGGACACGGTGGAGGACTCCTTCATCTCCGGCGTGCTCGAGCGCCGCCA

365   CCCACTTCCGAAGACCTCCGATCCGGCAGTCCAGATTTCCGGTAACTTCGCT

417   CCGGTGGACGAGCACCCGGTGCAACACCACCTTCCCGTCTCCGGCCGCATCC

469   CACGGTGCCTTGACGGCGTCTACCTGCGCAACGGCGCCAACCCACTCCTTGA

521   GCCCGTAGCCGGCCACCACTTCTTCGACGGCGACGGCATGGTCCACTCCGTC

573   AGCCTCCGACAGGGAACCGCCAGCTATGCCTGCAGGTTCACCGAGACCCATC

625   GGCTAGTGCAGGAGCGTGCGATCGGCCGGCCGGTCTTCCCCAAGGCAATTGG

677   CGAGCTCCACGGCCACTCCGGCATCGCCCGCCTCCTCCTCTTCTACGCTCGC

729   ACTGCTACCGGCCTCGTCGACGGCTCCAGCGGAACCGGCGTCGCCACCGCTG
                                JZ184
781   GTCTGGTCTACTTCAACCGGCACCTCCTCGCGATGTCCGAGGATGACCTCCC
                          JZ185
833   CTACCACGTCCGGGTCACCTCCTCTGGCGACCTCGAGACCGTTGGCCGGTTC

885   GACTTCATGGGTCAGCTCAATTCCGCCATGATTGCCCACCCCAAGCTCGACC

937   CGGCTTCGGGCGAGCTCTTCGCTCTCAGCTACAACGTCATCAAAAAGCCATT

989   TCTCAAGTTCTTCAAATTCACCTCAGATGGAAAGAAGTCCCCAGACGTCGAA
                                      JZ186
1041  ATCCCCATTGATCAGCCCACCATGATTCATGACTTCGTCATTACTGAGAATT
      JZ187
1093  TCGTCATAATTCCAGACCAGCAGGTGGTTTTCAAGCTCCAGGAGATGATCCG

1145  TGGTGGCTCTCCGGTCGTCTATGACAAGAAGAAAACCGCCCGGTTCGGAATT
```

1197 CTATTGAAAACCGCTGCCGACTCGAACGGTCTGAGGTGGATTGACGCGCCGG

1249 ACTGCTTCTGCTTCCACCTCTGGACCGCCTGGGAAGAACCCGAAACCGACCA

1301 GGTCGTCGTCATCGGCAGCTGCATGACGCCGCCGGACTCGATCTTCAACGAA

1353 TCCAACCAGAGCCTGAAAAGTGTTTTGACTGAAATCCGGCTTAATTTGAAAA

1405 CCGGCCTGTCGAGCAGGAGGGAGATCGACCCATCAAGGCACTTGAATTTGGA

1457 GGTTGGGATGGTGAACCGGAACCGGCTCGGCAGAGGACCCGGTGTGTCGCTA

1509 TCTAGCCATTGCCGACCGTGGCCAAAGGTGTCCGGGTTCGCCAAGGTGGACC

1561 TCTCGACCGGGGAGGTGACCAAGTTTATCTACGGCGAACAGTGCTATGGCGG

1613 CGAGCCATACTTTGTGTCCAGAGATCCGGTGGCGCCGGAGGACGATGGGTAT

1665 GTTCTGTCGTTCATGCACGACGAGAAGACGGCGCGATCAGAGCTGCTGATCG

1717 TCAATGCCATTACCATGCAGCTAGAGGCCTCTGTGAAGCTCCCATCCAGGGT

1769 CCCCTATGGATTCCATGGGACTTTCATCAGTAGTAAGGACCTTGCAAATCAG

JZ223

1821 GCCTGAGTCGACTCAGTGAGTCGCATCGGAGCCTATCTGCATTTCATCAATC

JZ222

1873 ATAGCATCTTTTTAAAGGAGTGGAGATTATACCTGAGGGATATGATTAGGCT

1925 TGCGTCCCCGGGATCTCCTTCCTTAGATGACCCATTTCGGGTTTTTAGCTTT

1977 TGTCTCTCCGCCAGTTACCACGGCCAGTGTGAGAGAAACCAGCTTGAAGCTT

2029 TGGGTGTAGATAGGTTTGGAGCTCAGCTGTTTCTCTGTAATTCTTTTGTGTT

2081 GTGCATGAATTGGATGTAATGTATGTATGTAATTGGGAAAGTGAGGTCTCTG

JZ224

2133 GGATCCCCTTCCCTTCTTCCACTGCTTGTATATATAATAAAAAGGCTACACC

2185 TTTCGCCCCAAAAAAAAAA

Nucleotide Sequence of *PaNCED2*

JZ153

1 CCATTAACGAACGAATCTGCGCAGGCATTTCCGTTTTTCAAGAAAAACAGAG

53 AATAGAGAATACAGAGAGAGGGTGTGTGTTCATAATAAGTAGGGCATACACA

105 CCAGACACTAGAAAAAAACTGAGAGGAGAAGAGGAAGAGGAAGAAACCAATC

157 AGAGCAGACT**ATG**CAGAAGGAGAATGAGAAGAAGAAGATCGTGATCCGTGAT

209 CCGAAGCCGACTAAGGGATCGTATCGCGATGGTGGACGCATGGAGAAATTGA

261 TAGTGGGTCCATGCCTACTCTCCGGGAATTTCCCCCTGCGGGTCGAAGAAAC

313 TCCCTGCGAGAATCTCCCCATCAAAGGTACCTCCCGGATTGCTTGGATGGGG

365 GAGTTTGTTAGAGTGGGCCCGAACCCGAAGTTTGCTCCTGTGGCTGGATATC

417 ATTGGTTTGACGGTGATGGCATGGTTCATGGAATGCGTATTAAAGATGGAAA

469 AGCAACCTATGTTTCACGTTACGTGAAGACATCTCGCCTTAAACAAGAAGAG

521 TATTTTGGAGGAGCAAAATTTATGAAGATTGGAGACCTTAAGGGCATGTTTG

573 GGCTTTTTATGGTTAACATGCAAATGCAAATGCTTCGAGCAAAACTCAAAGT

625 GCTGGACGTTTCATATGGAACTGGGACAGGCAATACAGCTCTAATATATCAT

677 CATGGTAAACTATTGGCCCTTTCAGAAGCAGACAAACCTTATGTCCTTAAGG

729 TTCTGGAAGATGGCGATCTTCAAACCCTTGGGATGTTGGATTATGACAAGAG

781 ATTGTCTCATTCATTCACTGCTCATCCCAAGGTCGACCCATTTACTGATGAG

833 ATGTTACATTTGGGTATCTCCCATACACCATACTTAACGTACCGCGTTATAT

885 CCAAAGACGGCATCATGCACGATCCAGTCCCCATAACAATACCAGAAGTCAT

JZ121

937 GATGCATGATTTCGCAATAACTGAAAACTATGCAATCTTCATGGATCTTCCT

989 TTGTACTTCCGACCGAAGAAAATGGTGAAGGGGAAACTTATCTTCCCATTTG

1041 ATGCAACAAAGAAAGCTCGTTTTGGTGTACTACCACGATATGCAAAGGATGA

1093 ACTTCAAATGAGATGGTTTGAGCTCCCAAATTGTTTTATCTTCCATAATGCT

JZ120

1145 AATGCTTGGGAGGAAGGTGGTGAAATTGTTCTAATCACTTGCCGCCTCCAGA

JZ148

1197 ATCCGGGCCTGGACATGGTCAGTGGAATCGTGAAAGAGAAGCTTGAAAATTT

JZ147

1249 CAAAAATGAGCTGTATGAAATGAGGTTCGATATGAAAACTGGTGCTGCTTCT

1301 CAGAAACAATTATCGGTATCTGCTGTAGATTTTCCCCGGATCAATGAGTCTT

1353 ACACAGGCAGGAAACAACGGTTTGTCTATGGAACCATACTCAACAACATTAC

1405 AAACGTGAAGGGCATCATCAAGTCTGATCTGCAGCTGAACCAGAGGGACGGA

1457 AATCGAAGCTCGATGTTGGAGGTAATAATCAAGGCATCTTTATCTTTCAGTT

1509 GGGACCCGGGGTTTGTTCCTCGGAAGCCTGGTGTCACTTCAGAAGAGGATGA

1561 CGGCTATTTGATATTCTTTGTACTGACCGAACGGACTGGAAAATCCGAAGTT

1613 AATGTAATCGATGCGAAAACAATGTCAGCTGATCCTGTTGCGATTGTGGAAC

1665 TGCCCCATAGAGTTCCATTTGGGTTTCTGCCCTTCTTTGTATCAGAGGAACA

JZ161

1717 ACTTCAACAACAGGCAAAGATCTAATACTGCTTTCAAGTTCTGGCTGATATT

1769 ACTGGTAGGAGTATTGACTGCATTGCCCAGGATGAGTAAAGAGTTTCTTATT

1821 GTCTGTAAGATGGAAGCCACTGATATAGGTACTGTATTCATATAGAACTATG

1873 AAGGGAAGGCTTGAGATTGTACTAAGATATATGATCTTGATTTGCTCGAGCA

JZ162

1925 AATAAAACTACGTAAACTTTTGAGTTCCCAAAAAAAAAAAAAA

Nucleotide Sequence of *PaNCED3*

JZ250

1 CTGAGAGAGACAGCTCTTCAAATCCAATACTCTTCGATACTCTTATGTCAAT

53 GGCTACTCCTACTACTACTTGTGGGGCAGGTGATCTACTTCAAAATCCCAAA

105 TTGCTCCCCATTTCAAAGAATCTCAGCCGTCCAAAAAACTTCATCATGCTAA

157 AACACAACACCCCATTAATTCAGTGCTGCTCACATTCTCCTTCTTCTTCTTC

209 TGCTGCTGTCCTTCATCTACCACCAAAGCAGCCGACAAAATCCAAACCGTCC

261 ATCAAGAAAGGAGAGAAATCGTCGACTCTCACTCCATCGATAGAGAAGAATC

313 CTGGCAGCCATCAAGTGAAAACAGATCAATCGGGTCCGAACCGGGTCGGACC

JZ245

365 CAACTGGAACATTTTTCAACGGACTGCTGCCTTCGCCTTGGACGCGATCGAG

417 GAGAAACTCATTGCTCGGGTGCTCGAGCGCCGCCACCCGCTTCCAAAGACCG

469 CGGACCCGGAGGTTCAGATTGCCGGAAATTTTGCACCGGTCGCCGAGCATCC

521 TGTACAGCACGGCATCCCCGTCGCCGGAAGAATTCCTCGCTGTCTCGACGGC

573 GTCTACGTCCGCAACGGTGCCAACCCCTTGTTCGAGCCGATCGCCGGCCACC

625 ATTTCTTCGATGGAGACGGGATGATCCACGCCGTCCGGTTCCGAAATGGGTC

677 CGCGAGTTACTCTTGCAGGTACACCGAGACTCGGAGGCTGGTGCAGGAGCGC

729 CAGCTCAGCCGGCCGATTTTTCCGAAGGCTATCGGCGAGCTGCACGGCCACT

781 CTGGCATCGCCCGCCTCCTTCTCTTCTATACAAGAGGGCTGTTTGGGCTGGT

JZ240

833 GAACGCCGACGAGGGAATGGGAGTAGCCAACGCCGGTTTGGTCTACTTCAAT

885 CGCCGCCTCCTCGCTATGTCTGAGGATGACCTCCCCTACCACGTCCGCATCA

937 CCCCGTCCGGCGACCTGAAGACCGTCGGACGACACGACTTCGACAACCAGCT

989 CCGCTCCTCCATGATCGCCCACCCCAAGCTCGACCCAGAATCGGGCGAGCTC

1041 TTCTCCCTCAGCTACGACGTCGCCCGAAAGCCTTATCTGAAATACTTCCACT

1093 TCGCCCCCGACGGCTGGAAGTCGCCGGACGTCGAGATCCCCCTCGACAGGCC

1145 GACCATGATCCACGACTTCGCCATTACCGAAAACTTCGTCGTGATTCCCGAC

1197 CAACAGGTGGTCTTCAAGCTAGAAGAGATGATAAGAGGGGGCTCTCCGGTCG

1249 TCTACGACAAGAACAAGACCTCCCGATTCGGAATTCTCCCGAAATACGCCCC

1301 CGACGCGTCGGAAATGATATGGGTCGACGCCCCGGACTGCTTCTGCTTCCAT

1353 CTCTGGAACGCGTGGGAGGAGCCGGAGTCCGGCGAGGTGGTGGTAGTCGGCT

1405 CGTGCATGACGCCGCCAGACTCAATATTCAATGAAAACGAGGAGAGCCTGAA

1457 GAGCATTCTAACGGAGATCCGGCTCAACACGAGGACAGGTGAGTCGACTCGC

1509 CGGACCATCATCGACCCGCAGAAGCCGTTGAATTTGGAAGCTGGGATGGTGA

1561 ACCGGAATCGTTTGGGGAGGAAGACCCGGTTCGCGTATCTTGCCATTGCAGA

1613 GCCGTGGCCGAAGGTGTCGGGTATCGCGAAGGTGGATCTTGGGACGGGGGAG

1665 GTGAACCGGTTTGTGTATGGGGAGAGGCAGTTCGGTGGAGAACCGTATTTCA

1717 TTCCGAGAGAGCCGAGTACGTCGGGACGAGAGGACGACGGGTATGTGGTGTC

1769 GTTCATGCATGACGAGAAGACGTCGAGGTCTGAGCTGCTTATCTTGAATGCA

1821 ATGAATATGAGGTTGGAGGCGTCTGTGATGCTTCCTTCCAGAGTCCCATATG

1873 GATTTCATGGCACTTTTATTAGTTCCAGGGACCTTGCAAAACAAGCC**TGA**CA

JZ225

1925 CAGATCGGAGAGAGGAACAAGACCCTGAGACTGAGTCGGAATCGTCTTCGTC

1977 TTCTTCTTCTTCTGATTATTATTAGTGTTGTTATTGTTGTTATCTTTTTTTA

2029 AGGGGAGATAATACCAGGGGGATGAGTGGAGCTACGTCCCCGGAGTCTTCTC

2081 TTTTGCTATTACAGGCCTTCTACGTTTGTGGGTTTTACGTGGTTTCTCTCTT

JZ206

2133 CTTCTTCTGCTTCTTTAAATATCTTTAATTTGTTTGTTTTAGGTGTGGAACC

JZ205

2185 AGCTCGAAGCTTGTTAGATTGTAGGTAGATTTGTACCTTAGCTGGTTCTCTA

2237 TAATTATTTTACTCTAGTGTGAATGAATTTATGATCTTACTAGTGGTTACTA

2289 TGCAAAGAAAAAAAAAAAAAA

Southern Analysis.

In order to assess the number of genes present in the avocado *NCED* family, Southern blot analysis was performed using the three full-length genes as probes (Figure 3.3A-D). High stringency washes of Southern blots probed with full-length probes of either *PaNCED1* (Figure 3.3A), or *PaNCED3* (Figure 3.3B) revealed that there was little cross-hydridization of the two genes under these conditions. The hybridization signals seen on the autoradiogram corresponded to those predicted based on the restriction map for the two genes.

Southern blots of *PaNCED2* washed at low stringency produced several bands in each lane digested with different restriction enzymes (Figure 3.3C). Based on the restriction enzyme map of *PaNCED2*, the strong signals (two bands are present in the *Bam*H1 lane, two in the *Eco*R1 lane and two in the *Hind* III lane), correspond to *PaNCED2*. The signals that cannot be explained from the sequence of *PaNCED2* are likely due to cross-hybridization with related sequences in the genome. When the same blot was washed at high stringency (Figure 3.3D), several faintly hydridizing bands still remained, particularly in the lane of the *Hind*III digest. The fainter bands still visible after high stringency washing are likely to be due to related members of the *NCED* gene family. Low stringency washes of blots probed with either *PaNCED1* or with *PaNCED3* also produced multiple bands (data not shown).

Monitoring of the Ripening Process.

Avocado fruits left at room temperature require, on average, about two weeks to become fully ripe. Some variation occurs between varieties (Biale and Young, 1971). Lula produced little ethylene until six days after harvesting, at which time there was a

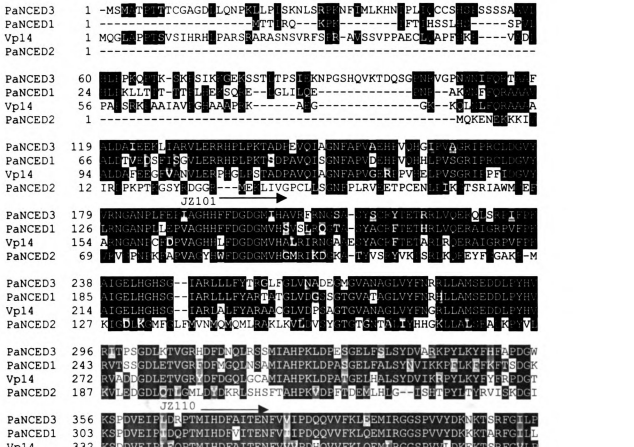


Figure 3.2. Alignment of the deduced amino acid sequences of PaNCED1, PaNCED2 and PaNCED3 from avocado with VP14 from maize. Amino acid residues identical in at least three of the sequences to the VP14 sequence at a given position are indicated by black boxes. Grey boxes indicate amino acid residues that are conserved in at least three of the four sequences. The arrows indicate the regions that were used in the design of the degenerate primers.

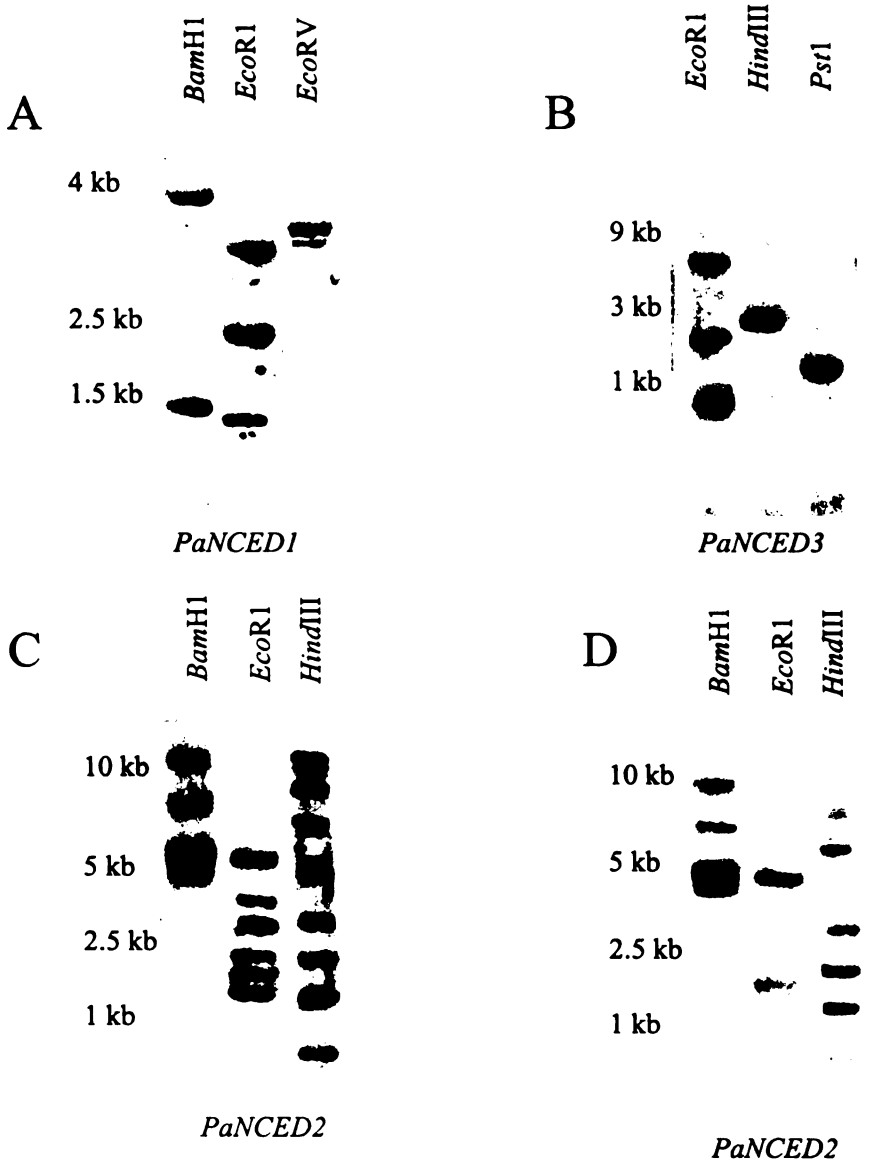


Figure 3.3A-D. Southern blot analysis of genomic DNA isolated from leaves of avocado, cv. Lula. Each lane contained 30 μg of DNA digested with the indicated restriction endonucleases. A) Analysis of *PaNCED1*. The blot was probed with a random-primed labeled probe (2.2 kb) corresponding to the full-length gene and washed at high stringency (see Materials and Methods). *PaNCED1* contains a restriction site for *Eco*R1 and *Bam*HI, but not for *Eco*RV. B) Analysis of *PaNCED3*. The blot was washed at high stringency. *PaNCED3* has two restriction sites for *Eco*R1, which are approximately 600 bp apart, and does not have restriction sites for either *Hind* III or *Pst*I. C,D) Analysis of *PaNCED2*. The blot was probed with the full-length gene (1.9 kb) and washed under low stringency. After development of the autoradiogram, the same blot was washed further under high stringency (D). The two strong signals present in the *Bam*H1 lane are consistent with the restriction map of *PaNCED2*, as are two of the signals seen in the *Hind*III digest, and the strong signal of approximately 5 kb in the *Eco*R1 digest.

massive increase in ethylene production (Figure 3.4A). This autocatalytic ethylene production is typical of climacteric fruit and other senescing tissue (Brady, 1987). By day 10, when ethylene production had declined, the fruit had a soft texture and fruit maturation was complete. In fruit ripened for six days, ethylene production had peaked while ABA levels remained at a low level. By day 11, four days following the peak in ethylene production, ABA levels had reached 30-fold higher levels compared to the level in unripe fruit. Because the enzyme involved in producing ABA would already be present by day 11, an earlier time point, namely, day 8 fruit, was chosen as the RNA source used in RT-PCR.

Northern Blot Analysis of the Three Genes.

In maize, there is an increase in transcript level of *Vp14* in leaves subjected to wilting (Tan et al., 1997). It was hypothesized that, as in water-stressed leaves, the increase in ABA levels during fruit ripening may also be accompanied by an increase in the mRNA levels of the *NCED* genes. Northern analysis of *PaNCED2* showed that it remained fairly constant in expression during the ripening process (Figure 3.4B). For analysis of *PaNCED1* and *PaNCED3*, the same blot was stripped and reprobed with gene specific probes based on the 3' non-coding region of each of the genes. For *PaNCED3*, the gene specific probe was amplified using primers JZ225 and JZ205. The PCR fragment produced using these primers corresponds to the region from position 1939 bp to position 2239 bp. For *PaNCED1*, a gene specific probe was amplified using primers JZ223 and JZ224. This product corresponds to the region extending from 1827 to 2162 bp. Control experiments revealed that each of these probes did not cross-hybridize with the other two genes (data not shown).

Both *PaNCED1* and *PaNCED3* were barely detectable until 8 days after harvesting. At this time, mRNA levels of both *PaNCED1* and *PaNCED3* increased in a similar fashion reaching the highest levels at day 10, and falling again as the fruit became very soft (12 days). Since this increase in message levels precedes the increase in ABA levels, both *PaNCED1* and *PaNCED3* can be viewed as possible cleavage enzyme genes.

To test whether the avocado genes cloned from fruit were up-regulated during wilting of leaves, Northern analysis was performed on turgid avocado leaves, and leaves that had been wilted to 95, 88, and 80% of their fresh weights. As a result of the dehydration, ABA levels increased approximately 10-fold in leaves that lost 20% of their water content (Figure 3.5A). While *PaNCED3* was undetectable under any of these conditions, *PaNCED1* increased dramatically in response to water loss (Figure 3.5B). *PaNCED2* remained fairly constant under the same conditions.

Chloroplast Import Assays.

Carotenoids are found associated with the photosystem II light-harvesting complex of the thylakoid membrane, and in much smaller quantities, with the chloroplast outer membrane (Siefermann-Harms et al., 1978). Any enzyme that utilizes carotenoids as substrates must, therefore, be imported into the chloroplast. An example of this is zeaxanthin epoxidase, which has been shown to be targeted to the thylakoid membrane (Marin et al., 1996). Proteins destined for the chloroplast are synthesized as precursors with a serine/threonine-rich domain lacking in acidic amino acids at the amino terminus (VonHeijne et al., 1989). Within the chloroplast, the signal peptide is cleaved by a protease, and the protein targeted to the thylakoid membrane (Keegstra and Cline, 1999). Sequence analysis indicated the presence of a targeting domain in the deduced

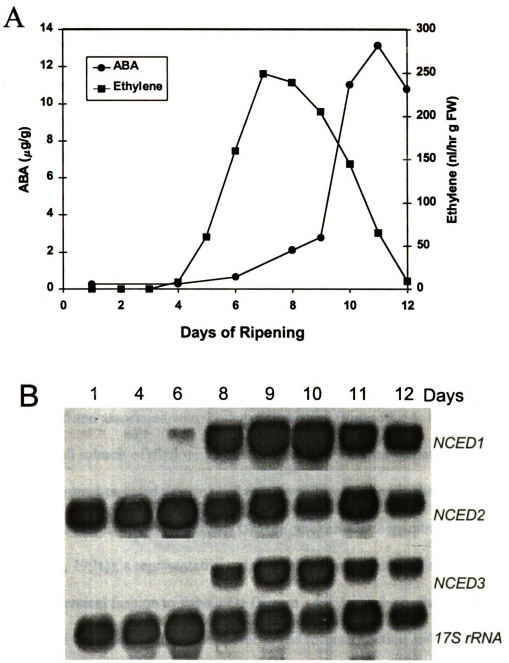


Figure 3.4A-B. Changes in ABA, ethylene levels, and in *PaNCED1, 2* and *3* transcripts accumulation during the course of fruit ripening in avocado. A. Analysis of ABA and ethylene levels plotted as a function of days of ripening. B. Northern analysis of *NCED* gene expression in the same fruit. Total RNA (30 μg per lane) was isolated from fruit, electrophesized and blotted onto nylon membranes. The same blot used for analysis of *PaNCED1* was stripped and reprobed with probes against *PaNCED2* and subsequently *PaNCED3,* and 17S rRNA . *PaNCED1* and *PaNCED3* increased as the fruit ripened while *PaNCED2* remained constant. The specific probes used for the *PaNCED* genes are described in Materials and Methods. The 17S rRNA probe was used as a loading control.

amino acid sequences of both PaNCED1 and PaNCED3, and the absence of such a domain in PaNCED2 (Emanueffson et al., 1999). Therefore, the avocado NCEDs were tested for *in vitro* import into isolated pea chloroplasts.

Chloroplastic proteins carrying a transit peptide require ATP for their binding and translocation (Olsen et al., 1989). Radiolabeled PaNCED1 was incubated with pea chloroplasts either in the absence of ATP or at low ATP levels (Figure 3.6A, lanes 1 to 8). Following fractionation of the chloroplasts into pellet and stromal fractions, PaNCED1 was found in the pellet fraction. When ATP concentrations were raised to levels that are conducive to translocation of bound precursors (Olsen et al., 1989), [^{35}S]PaNCED1 and chloroplasts were fractionated, PaNCED1 was not found in the stromal fraction, and did not undergo a decrease in molecular weight. Thus, PaNCED1 was not imported and associated with the outer membrane (Figure 3.6A, lanes 9 to 12). In contrast, the small subunit of ribulose bisphosphate carboxylase/oxygenase (prSS), a representative stromal-targeted protein used as a control, was found in the stromal fraction (Figure 3.6B, lanes 7-10). Further, OM14 (outer envelope membrane protein of 14 kDa (Li et al., 1991)), a representative outer envelope protein used as a control, behaved in an identical fashion to PaNCED1 (Figure 3.6B, lanes 1-4). PaNCED1 fractionated with the pellet fraction as opposed to the stromal fraction and did not undergo a decrease in size, indicating that no processing had occurred.

When chloroplasts from the import assay of PaNCED1 (with the bound PaCED1), were treated with thermolysin, (indicated by 'post' in Figure 3.6A, lanes 1-12), no protected protein fragments bands were observed. This suggests that if, in fact, PaNCED1 is inserted into the membrane, it is oriented such that it is accessible to the added protease

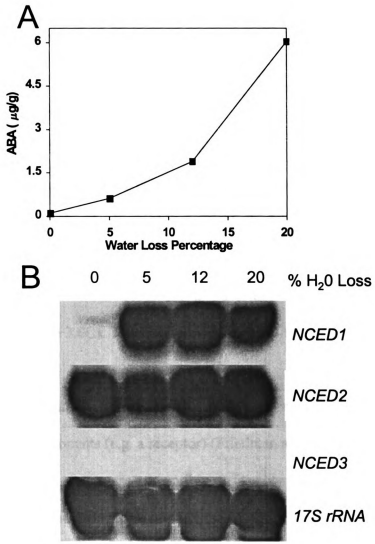


Figure 3.5A-B. Accumulation of ABA (A), and of *PaNCED1*, *PaNCED2*, and *PaNCED3* transcripts (B) in response to wilting of avocado leaves. RNA blot hybridizations were carried out with total RNA (30 μg per lane) isolated from leaves that had been wilted to increasing percentages of their fresh weights. The leaves were wilted using a pressure bomb for approximately 15, 30, and 50 min to achieve water losses of 5, 12 and 20%, respectively. After this time, the leaves were incubated in the dark for 4 h, and then frozen in liquid nitrogen. The specific probes used for the *NCED* genes and the probe (17S RNA) used as a control are described in "Materials and Methods".

and thus likely faces the cytosolic side. This result is identical to that found with OM14, which is also not protected by post-thermolysin treatment of import reactions (Figure 3.6B, lanes 1 to 4), and contrasts to prSS, which is protected from thermolysin treatment after import (Figure 3.6B, lanes 7-10).

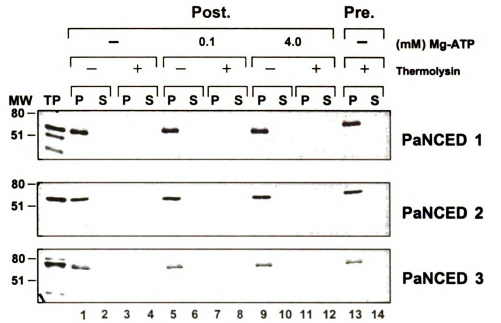
To determine whether the bound [^{35}S]PaNCED1 was peripherally or integrally associated with the outer membrane, chloroplasts were subjected to NaCl and sodium carbonate extraction after incubation with PaNCED1. Such treatments, which remove peripheral membrane proteins, were ineffective in extracting PaNCED1 from the membrane, suggesting that it was at least partially assembled within the outer membrane of the chloroplast (Figure 3.6C). This behaviour is identical to that of OM14, used as a control (Figure 3.6C).

Chloroplast proteins carrying a transit peptide require one or more protease susceptible surface components (e.g. a receptor) (Friedman and Keegstra, 1986). To address the requirements of protease-susceptible surface components, chloroplasts were pretreated with thermolysin prior to an import assay. After thermolysin treatment, chloroplasts were repurified over Percoll, washed twice with import buffer containing 4 mM ATP, and incubated with PaNCED1. PaNCED1 could still insert into the outer membrane, indicative that no proteinaceous components (labeled as 'pre' in Figure 3.5A, last two lanes) were required for insertion. This result is identical to OM14 (Figure 3.6C, lanes 5 and 6), which also does not require receptors to mediate its insertion into the chloroplast envelope (Li et al., 1991).

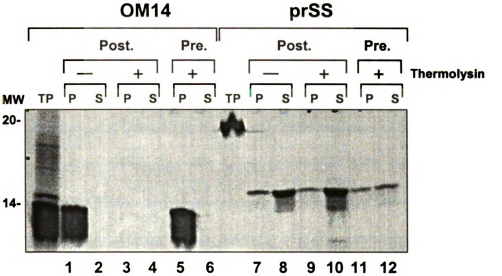
Chloroplast import experiments similar to those described for PaNCED1 were performed on PaNCED2. Under no or low ATP concentrations, PaNCED2 was bound to

Figure 3.6A-C. Chloroplast import analysis of PaNCED1, 2, and 3. Isolated pea chloroplasts were incubated with radiolabeled *in vitro* translation products, with either no (-) or low (0.1) ATP concentrations for binding, or under high (4.0) ATP concentrations for translocation. As control, prSS and OM14 were used. TP= total translation product. (A) Import reactions for PaNCED1, PaNCED2, and PaNCED3 under each of the ATP concentrations were separated into pellet (P), and stromal (S) fractions. Samples in lanes 3, 4, 8, 9, 11, 12 were treated with thermolysin *after* import, as indicated by a "+" above the lanes. Chloroplasts used for import in the final two lanes (13 and 14) were treated with thermolysin *prior* to import to discern the requirement for protein receptors on the surface of the chloroplast (indicated by "pre" above the lanes).
(B) Import analysis of a representative soluble protein (prSS), and an integral membrane protein (OM14). Under import conditions, radiolabeled mSS was found in the stromal fraction (S) and hence, was resistant to post-thermolysin treatment of the import reaction (lanes 7 to 10). OM14 was associated with the pellet fraction (P) following import, and was sensitive to thermolysin treatment of the import reaction (lanes 1 to 4). Pre-thermolysin treatment of the chloroplasts used in the import reaction, did not affect the binding of OM14 to the chloroplast membrane (lanes 5 and 6). The import of prSS (lanes 11 and 12), was greatly reduced under the same conditions.
(C) Extraction of the bound proteins from the chloroplast pellet fraction. Radiolabeled precursor proteins were incubated with chloroplasts under import conditions. The import reaction was scaled up over the standard reaction, as decribed in the Materials and Methods. After the import reaction, intact chloroplasts were re-purified and extracted with 100 mM sodium carbonate, or with 100 mM sodium chloride, or with 1% Triton X-100 and separated into supernatant (S) and pellet (P) fractions. Extraction of a representative integral, outer membrane protein (OM14) is included.

A


Post.
Pre.
–
0.1
4.0
–
(mM) Mg-ATP
–
+
–
+
–
+
+
Thermolysin
MW
TP
P
S
80
51
PaNCED 1
PaNCED 2
PaNCED 3
1 2 3 4 5 6 7 8 9 10 11 12 13 14

B


OM14
prSS
Post.
Pre.
Post.
Pre.
—
+
+
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+
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Thermolysin
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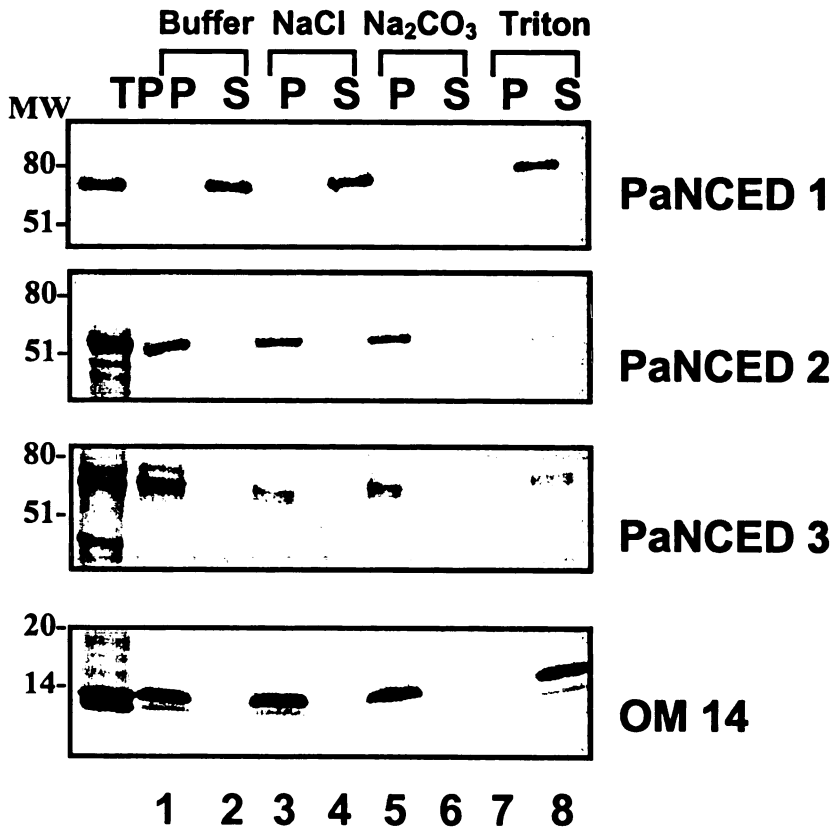
14-
1 2 3 4 5 6 7 8 9 10 11 12

C


Buffer
NaCl
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MW
T P P S P S P S P S
80
51
PaNCED 1
80
51
PaNCED 2
80
51
PaNCED 3
20
14
OM 14
1 2 3 4 5 6 7 8

the chloroplast membrane (indicated by lanes 1, 3, 5, 7, 9, and 11 marked 'P' in Figure 3.6A), but was not imported, as indicated by lack of protein in the stromal fraction. When ATP concentrations were raised, import still did not occur (Figures 3.6A, lanes 9-12). Instead, PaNCED2 remained associated with the pellet fraction, presumably in the outer membrane. After the import reaction, PaNCED2 was accessible to thermolysin (Figure 3.6A, lanes 4, 8, 12, indicated by a"+" above the lanes), and hence, faces the cytosolic side of the chloroplast. Extraction of chloroplasts containing the bound PaNCED2 with NaCl and sodium carbonate, or solubilization of the chloroplasts membranes with Triton X-100 was ineffective at releasing the protein from the membrane (Figure 3.6C). Thermolysin treatment of chloroplasts prior to import did not affect the insertion of PaNCED2, indicating that protein receptors are not required to mediate insertion.

The same import experiments were performed with PaNCED3, and similar results were obtained compared to PaNCED1, PaNCED2, and OM14. These can be summarized as follows: 1) Association of PaNCED3 with chloroplast membranes in the absence of ATP (Figure 3.6A, lanes 1 to 4), and failure to be imported into the stroma when ATP concentrations were raised (Figure 3.6A, lanes 9-12), 2) Degradation of PaNCED3 following treatment of import reaction with thermolysin (Figure 3.6A, lanes 3, 4, 7, 8, 11, 12), 3) Insertion of PaNCED3 into the membrane was unaffected by pre-treatment of chloroplasts with thermolysin (Figure 3.6A, lanes 13, 14), 4) Insensitivity of PaNCED3 to washes of the chloroplast membranes with either NaCl or sodium carbonate, or to solubilization of the membranes with Triton X-100 (Figure 3.6C).

Table 3.2. Comparison of Avocado *NCED* genes and their Encoded Proteins.

	PaNCED1	*PaNCED2*	*PaNCED3*
Total Message Length (bp)	2203	1967	2310
3' untranslated region (bp)	377	226	388
5' untranslated region (bp)	116	166	44
Total Coding Bases (bp)	1710	1575	1878
Number of Amino Acids	569	524	625
Predicted MW (kDa)	63.1	59.6	69.7
% Identity to VP14 (at amino acid level)	63	29	61
Isoelectric Point	7.4	9.0	8.2
Induction during Ripening	Yes	No	Yes
Induction during Wilting	Yes	No	No
Predicted Chloroplast Transit Peptide[a]	Yes	No	Yes
Localization[b]	OM[c]	OM[c]	OM[c]
In vitro ability to produce xanthoxin	Yes	No	Yes

[a] Based on the predictions of ChloroP (Emanuelsson et al., 1999)
[b] Based on the results of *in vitro* chloroplast import experiments
[c] O.M. = outer membrane of chloroplast

Assay of Enzymatic Activity of the NCED Protein Products.

The results of Northern analysis and sequence homology to *Vp14* supported a role for both *PaNCED1* and *PaNCED3* in ABA biosynthesis. In contrast, *PaNCED2* is constitutively expressed, and is less similar to *Vp14*. Therefore, *PaNCED2* seemed unlikely to be involved in ABA biosynthesis. To test whether the protein products of these genes could catalyze xanthoxin formation *in vitro*, all three genes were expressed as recombinant proteins fused to glutathione-S-transferase (GST). Although somewhat insoluble, the recombinant proteins were purified to homogeneity and used to assay for carotenoid cleavage. Both recombinant PaNCED1 and PaNCED3 cleaved 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin to produce xanthoxin and a C_{25} epoxycarotenoid (Figure 3.7). The reactions exhibited both protein (Figure 3.7A) and substrate (Figure 3.7B) dependency. *Trans*-isomers of violaxanthin and neoxanthin were not cleaved, consistent with the results of the VP14 assays, and with the required configuration for *cis*-ABA synthesis (Schwartz et al., 1997). The identity of xanthoxin (Figure 3.8), and the C_{25} compounds produced from either neoxanthin or violaxanthin was confirmed by mass spectrometry. Under the same assay conditions used for PaNCED1 and PaNCED3, PaNCED2 did not cleave either the *cis* or the *trans* isomer of either violaxanthin or neoxanthin.

Analysis of Carotenoid Composition of Ripening Avocado Fruit.

The carotenoid composition of fruit ripened for varying lengths of time was analysed to determine whether decreases in the levels of specific xanthophylls corresponded to increases in ABA. The carotenoids were identified on the basis of their acid-catalyzed shift in the absorption maxima. Lutein and lutein epoxide were the most

abundant carotenoids in unripe fruit, with levels remaining high in relation to the other carotenoids in fruit ripened for 10 days (Table 3.3). Between days 1 and 6, there was an increase in lutein epoxide, violaxanthin, neoxanthin and violaxanthin. As ripening continued (days 9 and 11), levels of these carotenoids decreased. The substantial decrease in neoxanthin that occurred between fruit ripened for 9 and 11 days is consistent with the increase in ABA levels that occurred during that time, but the two quantities cannot be related to one another on a 1:1 stoichiometric basis.

Table 3.3. Quantification of carotenoids from avocado fruit ripened for 1, 6, 9, and 11 days. Carotenoids were extracted from 1 g of fruit and purified using HPLC. The concentration of each carotenoid is expressed on a μg/g FW basis. Data are the mean of 4 measurements ± one SD.

Carotenoid (μg/g FW)	Days of Ripening 1	6	9	11
Lutein	2.65 ± 0.74	2.87 ± 0.56	6.49 ± 0.85	4.07 ± 0.76
Lutein Epoxide	2.88 ± 0.61	2.82 ± 0.64	1.68 ± 0.54	1.41 ± 0.43
Antheraxanthin	1.34 ± 0.33	1.36 ± 0.41	1.45 ± 0.40	0.93 ± 0.36
All-*trans*-Violaxanthin	1.13 ± 0.28	1.79 ± 0.47	1.23 ± 0.31	0.60 ± 0.23
9-*cis*-Violaxanthin	0.56 ± 0.19	0.78 ± 0.26	0.36 ± 0.13	0.27 ± 0.14
9'-*cis*-Neoxanthin	2.39 ± 0.73	3.75 ± 0.97	0.94 ± 0.36	0.74 ± 0.38

DISCUSSION

It is now becoming clear that under both developmental changes (fruit ripening) and physiological changes (wilting), ABA biosynthesis is regulated at the level of cleavage of C_{40} carotenoid precursors into xanthoxin. This paper is the first demonstration of the up-regulation of *NCED* genes during fruit ripening. The data support the circumstantial evidence derived from a variety of studies that implicated the cleavage reaction as the governing step in increasing ABA levels both in development and during wilting.

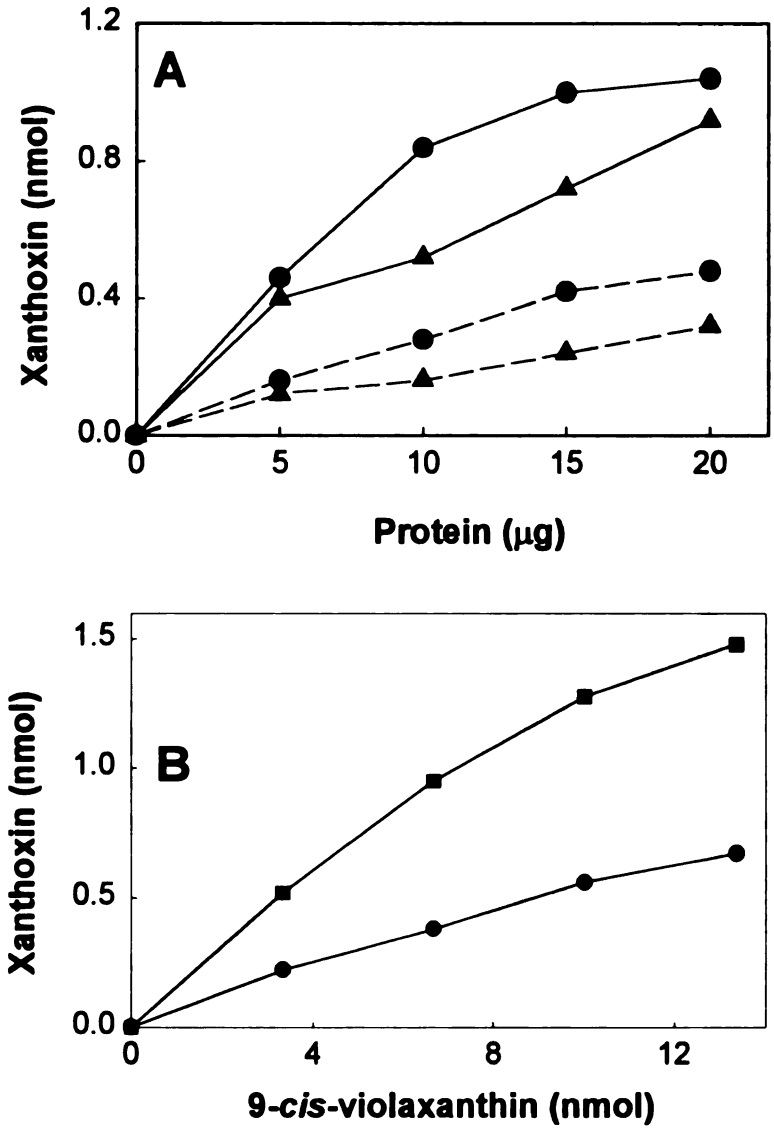

Figure 3.7A-B. (A) Increase in xanthoxin formed from either 9-*cis*-neoxanthin (▲) or 9-*cis*-violaxanthin (•) as a function of PaNCED1 (—) or PaNCED3 (---) protein concentration. Assays contained 6 nmol of substrate. (B) Xanthoxin formed by PaNCED1 and PaNCED3 as a function of 9-*cis*-violaxanthin concentration. The xanthoxin and C_{25} epoxycarotenoids produced in the *in vitro* reaction were analyzed by HPLC and identified by mass spectrometry.

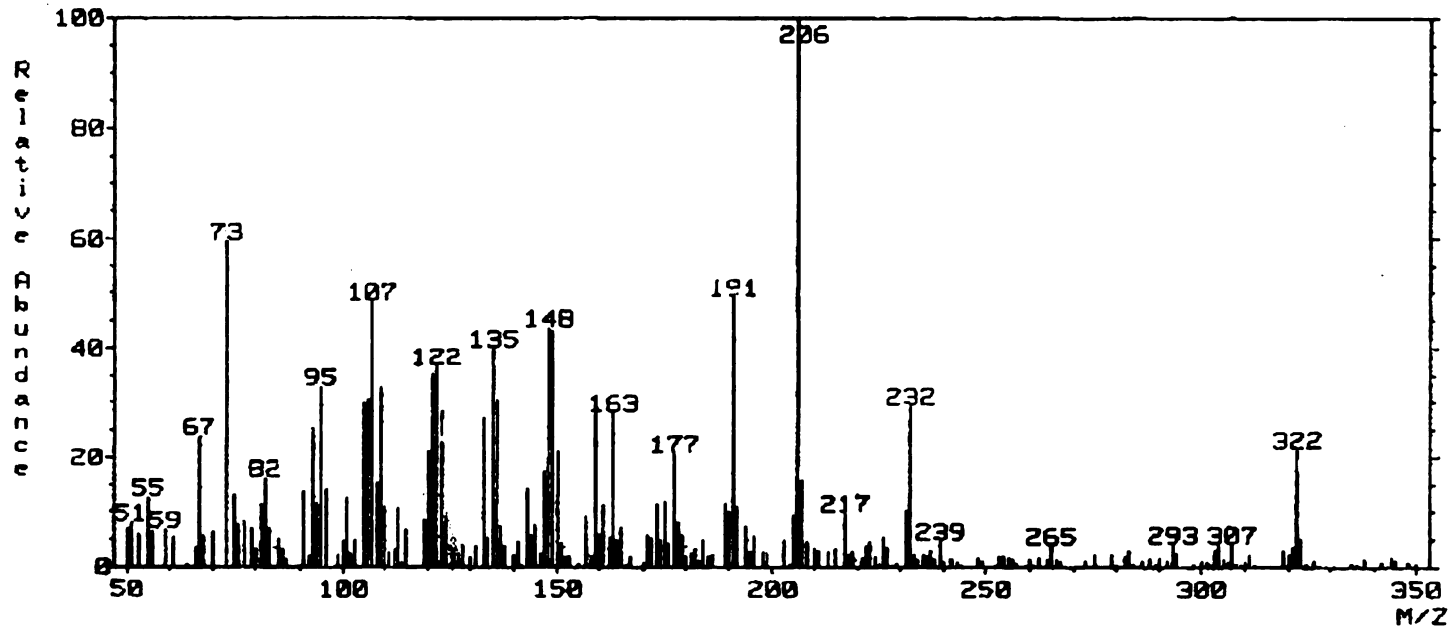

Figure 3.8. Mass spectrum of the trimethylsilyl derivative of xanthoxin produced from cleavage of 9-*cis*-violaxanthin catalyzed by *in vitro* expressed PaNCED1. This mass spectrum corresponds to spectrum 885 in Gaskin and MacMillan (1991), and has characteristic ions at 191, 206, 232, and 322 (M^+). Similar spectra were obtained using 9'-*cis*-neoxanthin as a substrate of PaNCED1 and for *in vitro* cleavage of 9'-*cis*-neoxanthin and 9-*cis*-violaxanthin catalyzed by PaNCED3.

The increase in ABA that occurs as avocado fruit ripens is substantial. However, the role that this increased ABA has in the fruit is not known. Many changes occur during ripening and the potential interactions between these processes are numerous (Brady, 1987). Presumably, ABA acts in fruit either by gene activation or by having a more direct effect, for example, by alteration of membrane properties. To determine the role that ABA plays in fruit, a system more amenable to transformation, such as tomato, may be useful. Antisense expression of the cleavage enzyme gene(s) may cause lowering of ABA levels, and the resulting effects on gene expression could be monitored. The 30-fold increase in ABA makes avocado a suitable fruit to study ABA biosynthesis. ABA mesocarp has also been used for other studies of ABA physiology (Lee and Milborrow, 1997).

In maize, *Vp14* is part of a multi-gene family (Tan et al., 1997). This is also the case in avocado. *PaNCED1* and *PaNCED3* are 60% identical at the amino acid level with *Vp14* and the tomato homolog, *LeNCED1* (Burbidge et al., 1999). Analysis of the sequence similarity of homologous sequences present in the database suggests that a large family of *NCED* genes exist. The genes can be grouped according to their identity to each other. For example, maize, bean, tomato, and avocado NCED1 and -3 share approximately 60% identity at the amino acid level to each other. PaNCED2 is approximately 60% identical to an *Arabidopsis* sequence, called *AtNCED1* (Neill et al., 1998), but only 30% identical to the aforementioned sequences. It would seem plausible that genes with 60% identity or greater may have the same function while those with less identity catalyze different reactions. The only proteins with demonstrated functions are the two avocado proteins described here, maize VP14, the bean protein, and lignostilbene

dioxygenase. The results of studies of the *notabilis* mutant (the mutant allele of *LeNCED1*), would suggest that the tomato gene product catalyzes the same reaction (Burbidge et al., 1999). The regions that are conserved among all of the protein sequences are likely part of the active site, and may be involved in cofactor binding. Site-directed mutagenesis would be useful in determining the function of the conserved residues.

The function of *PaNCED2* and similar *Vp14* homologs in other systems is not known. In *Arabidopsis*, a *PaNCED2* homolog called *AtNCED1* (60% amino acid identity) is moderately induced by rapid dehydration of leaves (Neill et al., 1998). We did not find up-regulation of *PaNCED2* during dehydration of avocado leaves. In addition, *PaNCED2* lacks a chloroplast targeting signal. It should be noted that proteins destined for the outer membrane of the chloroplast are not synthesized with an N-terminus extension, and possess a different import pathway in comparison with proteins destined for the thylakoid membrane (Keegstra and Cline, 1999). Since carotenoids are present in the envelope (Siefermann-Harms et al., 1978), a putative NCED need not be imported into the chloroplast for it to serve its function. Results of the *in vitro* import assay suggested that PaNCED2 is associated with the chloroplast outer membrane. However, immunolocalization will be necessary to confirm this.

Import analysis into isolated pea chloroplasts demonstrated association of all three proteins with the chloroplast envelope fraction. The interpretation of *in vitro* import experiments is complicated by the fact that the precursors utilized are from fruit, and the system used for import is pea chloroplasts. There is some evidence, for example, that import pathways for leucoplast proteins differ from those of chloroplasts (Wan et al.,

1996), and therefore one may predict that proteins in fruit may have a different import pathway into chromoplasts. However, avocado fruit differs from fruits such as tomato in that chloroplast structure is maintained and distinct thylakoid membranes are still present (Milborrow, 1974). Further, *PaNCED1* is expressed in leaves, and therefore is not a fruit-specific. Another consideration in *in vitro* import experiments is that the properties and folding characteristics of proteins affect their interaction with the translocation machinery. For example, hydrophobic proteins may insert into membranes randomly. Misfolded proteins may not have amino acid residues that are normally involved in interaction with a receptor protein exposed. Therefore, the results of the import experiments would need to be confirmed by other methods before drawing the conclusion that the three avocado proteins are localized to the outer membrane. To resolve the localization of the three proteins *in vivo*, antibodies designed against the three avocado proteins would need to be produced and immunolocalization and fractionation experiments performed.

The double bond present in both lignostilbene and in violaxanthin and neoxanthin is a common feature found in terpenoids, phytoalexins and many other natural products. Many of the biochemical pathways for these compounds occur in the cytoplasm. It is, therefore, possible that protein products of genes such as *PaNCED2* have no involvement in ABA biosynthesis or in carotenoid breakdown. The immunological detection mentioned above, as well as studies with reporter genes, such as the green fluorescent protein, may be useful in determining the localization of the enzymes with unknown function. Screening of T-DNA insertion lines of *Arabidopsis* may also be useful in making deductions about function.

Two avocado genes, *PaNCED1* and *PaNCED3*, encode proteins that are capable of *in vitro* synthesis of XAN, the precursor of ABA. Evidence for the *in vivo* role of *PaNCED1* and *3* in ABA biosynthesis is indicated by the correlation of mRNA levels of these genes with endogenous ABA levels. The chloroplast localization of PaNCED1 and PaNCED3 is also consistent with suppositions about the site of ABA synthesis (Zeevaart and Creelman, 1988). *In vitro,* PaNCED1 and PaNCED3 appear to be indistinguishable in terms of their substrate preference; both utilize violaxanthin more effectively than neoxanthin. Thus, ABA biosynthesis in fruit appears to be redundant, in the sense that two genes appear to encode proteins with identical functions. However, it should be emphasized that *in vivo* factors such as transport and degradation of ABA are also important in regulating levels. In addition, the *in vivo* accessibility of enzyme to the substrate, and the isomerization of violaxanthin into neoxanthin, may also be determinants of why two enzymes exist. It will be interesting to determine whether the presence of two very similar enzymes is typical of other plants as well, and if it occurs in organs other than fruit.

The carotenoid data (Table 3.3) indicate that there is a substantial decrease in neoxanthin as the fruit ripens. When expressed on a molar basis, the amount of ABA at any stage of ripening exceeds the amounts of both violaxanthin and neoxanthin. For example, late in ripening (day 11), the sum of the molar amount of violaxanthin and neoxanthin present is 2.7 nmol versus 49.2 nmol of ABA (derived from the data presented in Figure 3.4A and Table 3.3). This indicates that the carotenoid pool must turnover more rapidly than the ABA pool. In tissues such as light-grown leaves and in fruit, the ratio of carotenoids to ABA is very high, making it difficult to demonstrate

correlations between decreases in xanthophylls and increases in ABA. In roots (Parry et al., 1992) and in dark-grown, fluridone-treated leaves of *Phaseolus vulgaris* (Li and Walton 1990; Parry and Horgan, 1991), there is a 1:1 correspondence between xanthophylls cleaved and ABA + ABA catabolites synthesized. The decreases in both violaxanthin and neoxanthin that correlate with increases in ABA leves in fruit are consistent with their proposed role as ABA precursors, despite the lack of a 1:1 stoichiometric ratio.

Multi-gene families often encode genes with related functions, but in cases where the function is the same, differential regulation ensures that distinct genes are activated in response to different environmental and other stimuli. A good example of this is the ACC synthase gene family (Zarembinski and Theologis, 1994). The fact that *NCED* genes are part of a family can perhaps be construed as an indication that sensitive mechanisms are needed to regulate the amount and location of the ABA synthesized. In this regard, zeaxanthin epoxidase, an enzyme with both a structural and photoprotective role, does not exist as a gene family (Marin et al., 1996). For ABA to serve its role in drought response, a rapid signaling mechanism must be in place; perhaps the easiest way to achieve this is to have a specific signaling pathway to turn on the appropriate gene.

Differential regulation implies that different signal transduction pathways are activated that allow gene expression in response to the specific stimuli. Ultimately, the distinction between which genes are induced in response to a given stimulus lies in the promoter region. In avocado, two cleavage enzymes are present: both are induced during fruit ripening but only one of the genes is induced by water stress. Analysis of the promoter region of these two genes should reveal whether a dehydration response

element (DRE), as is found in many osmotic-responsive genes (Shinozaki and Yamaguchi-Shinozaki, 1997) is also present in *PaNCED1*. Promoter elements for genes that are up-regulated during fruit ripening include those that have an ethylene-responsive box (e.g. Itzhaki et al., 1994), those that have ripening-specific elements (e.g. Atkinson et al., 1998; Deikman et al., 1998), and others in which no previously characterized regulatory elements are apparent (e.g. Beaudoin and Rothstein, 1997). Comparison of the promoters of *PaNCED1* and *PaNCED3* may reveal which, if any, common *cis*-acting elements are present, and deletion analysis should indicate the role(s) that these elements play in regulating gene expression during fruit ripening and drought stress. It will also be interesting to determine whether other developmental cues, such as seed germination and embryo development, induce the expression of a novel *NCED*, and if overlap exists between the signal transduction pathways that lead to the expression of wilt-related *versus* developmentally-regulated *NCED* genes.

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Chapter 4

Identification of Cytochrome P450 monooxygenase genes using differential display

ABSTRACT

The rates of both biosynthesis and metabolism determine ABA levels. In many tissues, a major metabolic route of ABA is to the unstable intermediate 8' hydroxy-abscisic acid (8' OH-ABA), which rearranges to form phaseic acid. The enzyme that catalyzes this conversion, ABA 8'-hydroxylase, is a cytochrome P450 monooxygenase. Much evidence shows that ABA 8'-hydroxylase is regulated at the transcriptional level. Addition of exogenous ABA to suspension cultures can stimulate catabolism into phaseic acid.

A modified differential display approach was used to isolate cytochrome P450 monooxygenase genes that are differentially expressed in response to ABA treatment, and hence represent candidate genes for ABA 8'-hydroxylase. Differential display was carried out on RNA isolated from untreated suspension cultures and from cultures that had been treated with ABA, using degenerate primers designed against conserved regions of cytochrome P450 monooxygenase genes. Several bands were excised, reamplified, and cloned. These bands were tested by Northern analysis. Some of the cloned genes were not differentially expressed in response to ABA treatment and therefore, represented false positives. Some of the differentially expressed genes were not cytochrome P450 monooxygenases. Possible strategies to isolate the gene encoding ABA 8'-hydroxylase are discussed.

INTRODUCTION

ABA levels are regulated by the relative rates of biosynthesis and degradation. Large increases in ABA levels occur during wilting of leaves and during fruit ripening (see Chapter 3). In both of these cases, the increase in ABA is controlled by the rate of xanthophyll cleavage (see Chapter 3). Depending upon the tissue, ABA can be deactivated through two processes: catabolism and conjugation (Zeevaart, 1999). Catabolism occurs through oxidation of the 8' methyl group of ABA to form 8'-hydroxy-ABA (8'-OH-ABA), which is unstable and rearranges to form phaseic acid (PA). In addition to oxidation, conjugation of ABA by glycosylation or esterification may also be important in governing ABA levels in some tissues (Zeevaart, 1999). The pathway of ABA metabolism into phaseic acid via the unstable 8'-OH-ABA intermediate is shown in Figure 4.1.

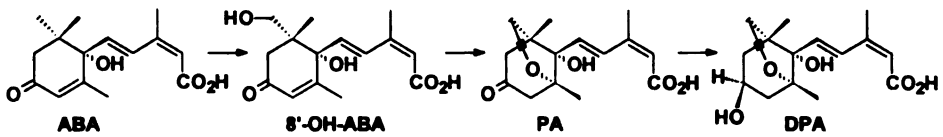

Figure 4.1. Pathway for the catabolism of ABA into phaseic acid (PA). The reaction of ABA into PA via 8'-OH-ABA is catalyzed by ABA 8'-hydroxylase, which is a cytochrome P450 monooxygenase.

Much evidence indicates that the enzyme that mediates the conversion of ABA into PA (ABA 8'-hydroxylase) is a cytochrome P450 monooxygenase. Cytochrome P450 monooxygenases are membrane-bound heme proteins that catalyze reactions in which two reducing equivalents from NADPH or NADH are transferred to the P450, and in which molecular oxygen is cleaved with one oxygen being incorporated in the substrate

while the other is reduced to water. Binding of the heme is through a cysteine residue situated between 15% from the carboxy terminus. A diagnostic test for cytochrome P450 involvement in a reaction is inhibition by carbon monoxide (CO), followed by reversal of the inhibition by irradiation with blue light (Bolwell et al., 1994). The overall reaction is shown below:

$$RH + O_2 + NADPH.H^+ \longrightarrow ROH + H_2O + NADP^+$$

A myriad of diverse cellular reactions involve the incorporation of an oxygen atom into the substrate. Reflecting this, cytochrome P450 monooxygenases are encoded by a highly divergent gene superfamily containing over 450 known cytochrome P450 sequences distributed among 65 gene families (Nelson, 1999). There are over 100 plant P450 cDNA/genomic DNA sequences that have been completed and that are distributed among 26 of these P450 gene families (Winkler et al., 1998). These sequences and other information about this class of enzymes can be found at the following web address: http://drnelson.utmem.edu/nelsonhomepage.html).

Numerous plant P450s have been cloned and/or biochemically purified. Some of the functions that have been ascribed to these P450s include certain steps in gibberellin biosynthesis, brassinosteroid biosynthesis, flavonoid biosynthesis, and herbicide detoxification (Schuler, 1996). However, for the majority of sequences, no function has been ascribed. A systematic attempt to assign functions to the *Arabidopsis* P450 sequences is being carried out by Winkler et al. (1998). In this approach, T-DNA mutants tagged in various P450 genes are being isolated, and examined for phenotypic effects.

The following lines of evidence support the notion that ABA 8'-hydroxylase is a cytochrome P450: 1) Incorporation of one ^{18}O from $^{18}O_2$ into PA (Creelman and

Zeevaart, 1984; Zeevaart et al., 1989), 2) Addition of the cytochrome P450 inhibitor tetcyclacis to *Xanthium* leaves prior to rehydration prevented PA accumulation (Zeevaart et al., 1990), 3) A cell-free system of *Echinocystis lobata* that was capable of converting ABA into PA was inhibited by carbon monoxide, and required oxygen and NADPH. (Gillard and Walton, 1976), 4) In stressed and subsequently rehydrated *Xanthium* leaves, CO reduced the accumulation of PA (Creelman et al., 1992), 5) A cell-free system prepared from maize suspension cultures catalyzed the conversion of ABA into PA. The reaction required oxygen and NADPH, and displayed blue light reversibility of CO inhibition (Krochko et al., 1998).

Transcriptional induction as a result of treatment with substrate is a common mechanism by which many cytochrome P450 monooxygenases are regulated. Specifically, in the case of ABA catabolism, several reports of induction of ABA 8'-hydroxylase by ABA exist: 1) Pretreatment of barley aleurone layers with ABA resulted in an increased conversion of [^{3}H]ABA into [^{3}H]PA (Uknes and Ho, 1984), 2) Potato and *Arabidopsis* suspension cultures pre-treated with 50 μM (±)-ABA exhibited an increased rate of [$^2H_6$]PA formation from [$^2H_6$]ABA (Windsor and Zeevaart, 1997), 3) A cell-free system from the embryonic axis of chickpea seedlings germinated in ABA had enhanced [^{14}C]ABA to [^{14}C] PA conversion (Babiano, 1995), 4) Maize suspension cultures treated with (+)-ABA exhibited 8'-hydroxylase activity while untreated cultures had no activity (Cutler et al., 1997).

As cytochrome P450s are membrane bound, and, in the case of ABA 8'-hydroxylase, of low abundance, biochemical purification appeared problematic. Therefore, the difference in activity observed in substrate-treated *versus* untreated

samples served as a basis to identify candidates for ABA 8'-hydroxylase. A molecular approach was undertaken involving differential display of untreated and ABA- treated materials and using degenerate primers designed against conserved regions of P450 genes. These regions used for primer design include the fingerprint heme-binding domain, present in all P450s, and two regions further upstream. These primers were used together with anchored oligo d(T) primers normally used in differential display. The use of degenerate primers in differential display in order to isolate specific classes of genes has been reported previously (Joshi et al., 1996). Recently, a similar approach was used successfully to isolate differentially-expressed cytochrome P450 monooxygenase genes from elicitor-treated soybean suspension cells (Schopfer and Ebel, 1998).

MATERIALS AND METHODS

Plant Material.

Five- to seven-day-old *Arabidopsis thaliana* or potato suspension cell cultures were maintained at 25°C in the dark. For treatment of the cultures, (±)-ABA was added to a final concentration of 50 μM. At various times after the addition of ABA, aliquots of the culture were removed, filtered, and washed with fresh medium. The tissue was frozen at -80°C for later use in RNA isolation.

RNA isolation and Northern analysis.

RNA was isolated by the method of Vanderlerberge et al. (1992). For a description of cDNA synthesis, see differential display and cloning (next section of Materials and Methods). Northern analysis was carried out by electrophoresis of 30 μg total RNA on 1.2 % (w/v) agarose gels containing 2.2% formaldehyde according to Maniatis et al. (1982). RNA gels were blotted onto Hybond H^+ membranes (Amersham).

Inserts to be labeled as probes were excised from the pGEMTeasy vector (Promega) using *Eco*R1. The DNA was purified using a Qiagen gel extraction kit and labeled with ^{32}P using random-prime labeling (Gibco-BRL). Prehybridization and hybridization of the membranes were performed at 65°C using Church-Gilbert buffer (Church and Gilbert 1984). The membranes were washed twice in 2x SSC, 0.1% (w/v) SDS at room temperature, followed by two washes in 0.2x SSC, 0.1% (w/v) SDS at 65°C.

Differential Display and Cloning.

RNA used as the treated sample in the differential display was isolated from cultures that had been treated with (±)-ABA for 6 h using the method described by Vanderlerberge et al. (1992). The method of Liang et al. (1993) was used for differential display with modifications as described in the GenHunter manual. All reagents used in the reverse-transcription and PCR reactions, with the exception of Taq DNA polymerase (Gibco-BRL), the degenerate primers (described below), and the (^{33}P)dATP, were from the GenHunter differential display kit. The main difference employed in the differential display technique employed here compared with the methods described by GenHunter is that the arbitrary primers normally used were replaced by degenerate primers that had been designed against conserved regions of cytochrome P450 monooxygenase genes. The sequences and relative positions of these three degenerate primers are shown in Figure 4.2. Each primer was used (at a concentration of 2 μM) in conjunction with the 3 anchored oligo d(T) primers (in this case oligo d(T_{11}). Conditions for reverse transcription were as follows: 65°C, 5 min; 37°C 60 min (after 10 min at 37°C, 1 μl MMLV reverse transcriptase was added to each tube), 75°C 5 min. PCR conditions were: 94°C, 30 sec; 40°C, 2 min; 72°C, 30 sec for 40 cycles, 72°C 5 min. The Genomx system

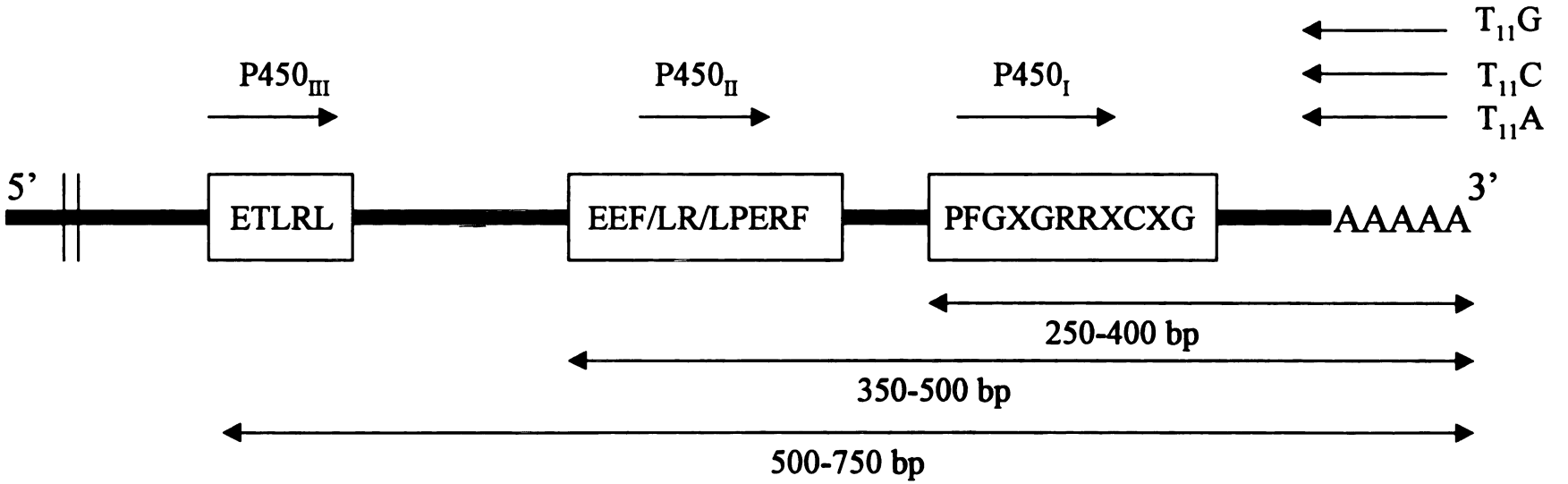

Figure 4.2. Schematic representation of the PCR strategy used to amplify cytochrome P450 monooxygenase genes. The domains present in many cytochrome P450 monooxygenases are indicated in boxes. The location of the forward ($P450_I$, $P450_{II}$ and $P450_{III}$) and reverse primers are indicated by the arrows. The primers were designed based upon the alignment of the deduced amino acid sequences of cytochrome P450 monooxygenase genes from a variety of plants. The sequences of the primers are as follows: $P450_I$: YWI HTI CCI TTY DSI I/KI/YI GGI I/MI/SI MG;
$P450_{II}$: GAR GAR TTY MGN CCN GAR MGN TTY; $P450_{III}$: GAR ACI YTR/I HWR/I YAC/I CC.
W=A/T, D=A/G/T, S=G/C, K=G/T,M=A/C, R=A/G, Y=C/T, N=A/G/C/T, H=A/C/T, I =inosine

was employed as the gel-running system, and reagents provided by Genomx were used in gel preparation. After overnight exposure of the gel, the autoradiogram was aligned with the gel. Bands present in the induced lanes were excised and eluted as described in the GenHunter manual. Reamplification of the eluted bands was carried out, and the purified PCR products were cloned into pGEMTeasy vector (Promega).

RESULTS

Differential Display of ABA-Treated Arabidopsis and Potato Tissue Cultures.

Sequence comparisons of cytochrome P450 proteins from animals, plants, and microorganisms have led to the identification of several conserved regions. For alignments of the amino acid sequences of several P450s, and for further discussion of these regions, see Frank et al. (1996); Vetter H-P et al. (1992). Three such regions were selected for the design of degenerate primers. The first region is the characteristic heme-binding domain that serves as a fingerprint for a cytochrome P450 monooxygenase (Bolwell et al., 1994). This region has been used extensively for the amplification of cytochrome P450 genes (Meijer et al., 1993). The other regions used in primer design are located closer to the amino terminus of a cytochrome P450 monooxygenase. These regions are designated the PERF domain and the ETLR domain according to the consensus amino acid sequence within that region (see Figure 4.2).

Differential display was carried out on cDNA prepared from *Arabidopsis* suspension cultures that had been treated with or without 50 μM (±)-ABA. The resulting differential display gel appeared similar to typical differential display gels obtained using arbitrary primers. A representative example of a differential display gel is shown in Figure 4.3. For each primer set used, different size products would be expected

depending on the distance between the region and the polyadenylation site. Based on a sequence alignment of several cytochrome P450 genes, the sizes of the various PCR products that were expected to be amplified with each of the primers are as follows: $P450_I$: 320-450 bp, $P450_{II}$: 400-600 bp and $P450_{III}$: 500- 700 bp. As few differences were seen between control and induced cultures when $P450_{II}$ and $P450_{III}$ were used as the primers; the subsequent analysis focussed on the differences observed when $P450_I$ was used as a primer.

In order to maximize the chance that the specific cytochrome P450 monooxygenase gene would be obtained as a differentially expressed band, different experimental systems were tried. Experiments similar to those performed with *Arabidopsis* cultures were also performed with potato suspension cultures. Bands that appeared to be differentially expressed in response to (±)-ABA treatment of the cultures were excised, reamplified and cloned.

In an attempt to increase the expression of ABA 8'-hydroxylase gene, *Arabidopsis* cultures were treated with trifluoro derivatives of ABA (Todoroki et al., 1994). The trifluoro derivatives are long-lasting analogues that have been found to give a greater increase in the rate of induction of ABA 8'-hydroxylase (Windsor and Zeevaart, 1997). Bands that appeared to be differentially expressed in these experiments were also excised, reamplified, and cloned.

A summary of the DNA fragments that were cloned from both the *Arabidopsis* and potato suspension cultures is presented in Table 4.1. Often each band was a mixture of more than one DNA fragment. To account for this, several clones that resulted from the cloning of each band were retained, as it was thought that they might represent

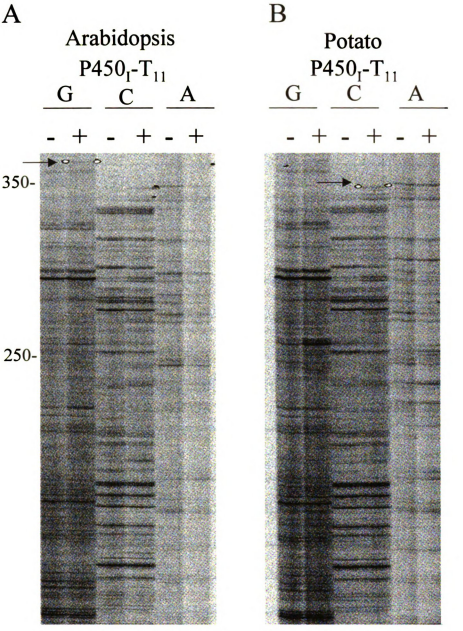

Figure 4.3. A) Differential display of RNA from untreated and ABA-treated *Arabidopsis* cell cultures. In this experiment, the primer designated P450$_1$, designed against the conserved heme-binding domain present in cytochrome P450 monooxygenase genes, was used in conjunction with three different sets of oligo d(T$_{11}$) 3' anchored primers (designated G, C and A). Bands that are present in the ABA-treated sample and absent in the untreated sample are indicated by dots. Size markers in base pairs are indicated on the left side of the autoradiograph. B) Differential display of potato cultures using the same method described.

Table 4.1. Summary of differential display bands that were amplified using the primer designated $P450_I$ and subsequently cloned from various tissues.

Band #	Plant species	Treatment	Anchor primer $H\text{-}T_{11}$-	MW (bp)
1	A.t.(susp)	(±)–ABA	A	400-450
2	A.t.(susp)	(±)–ABA	C	350-400
3	A.t.(susp)	(±)–ABA	G	320
4	A.t.(susp)	(±)–ABA	C	310
5	A.t.(susp)	(±)–ABA	C	290
6	A.t.(susp)	Tri-F ABA	G	650
7	A.t.(susp)	Tri-F ABA	C	400
8	A.t.(susp)	Tri-F ABA	A	400
10	A.t.(susp)	Tri-F ABA	A	350
11	A.t. (susp)	Tri-F ABA	C	325
12	Potato	(±)–ABA	C	325
13	Potato	(±)–ABA	C	320
14	Potato	(±)–ABA	C	310
15	Potato	(±)–ABA	C	310
16	Potato	(±)–ABA	G	320
17	Potato	(±)–ABA	G	270
18	Potato	(±)–ABA	A	500

[a] A.t.(susp) = *Arabidopsis thaliana* suspension cultures
[b] Tri-F ABA= 8',8',8'-trifluoroabscisic acid

different genes. One clone was then selected to be used as a probe to check for heterogeneity among the clones for each band. Based on the results of this screening, the number of individual clones to be tested on Northern blots was decreased. The PCR clones listed in Table 4.1 represent a single colony that was chosen from the cloning procedure, as opposed to a heterogenous mixture of bands that sometimes resulted from PCR amplification.

Several bands were sequenced prior to Northern analysis in order to assess the efficiency of the PCR primers in amplifying cytochrome P450 monooxygenase genes. Of a total of 18 bands sequenced, 10 were cytochrome P450 monooxygenase genes. The nucleotide sequences of these putative cytochrome P450 monoxygenases, as well as the sequences of other genes isolated by differential display are provided in the Appendix of this thesis. An alignment of the deduced amino acid sequences of some of the cloned cytochrome P450 monooxygenase genes with an *Arabidopsis* and a tobacco cytochrome P450 monooxygenase gene is shown in Figure 4.4. The region from the heme-binding domain to the end of the protein is presented, as this corresponds to the region of the genes that was amplified by the PCR reactions. The heme-binding domain is present in all of the identified sequences. In addition, several other conserved regions are evident within the carboxy terminus of the protein sequences.

The rate of success obtained in amplifying solely cytochrome P450 monooxygenase genes may not be as high as is observed in other PCR amplifications using degenerate primers. However, the primer used in this case ($P450_I$) was highly degenerate due to the lack of sequence conservation in that region. Further, the annealing temperature used in the PCR reactions was 40°C, so that non-specific products were

likely to be obtained (see Materials and Methods for the PCR conditions used). Northern blots of the identified cytochrome P450 monooxygenase genes are shown in Figure 4.5. Many of the bands listed in Table 4.1, when tested on Northern blots, did not show increased expression in response to (±)-ABA treatment, and thus were considered to be false positives.

Representative Northern blots for some of the genes that increased in response to ABA are shown in Figure 4.5. Because the membranes used in the Northern analysis were different for each of the clones, photographs of the RNA gels are included as loading controls. Each Northern blot shows expression of the particular PCR clone in reponse to increasing time of treatment of the suspension cultures with (±)-ABA. Genes listed as PCR Clones 2, 3, 5, 14, and 15 were either unexpressed, or expressed at low levels in untreated cultures, and the message levels increased in response to ABA treatment. These clones thus represented the true differentially-expressed genes isolated as a result of the procedure. The putative identification of these clones based on the results of searches for sequence homology of these clones is presented in Table 4.3 (also see Appendix). Clone 2 has no significant homology to genes in the database. Clone 3 is a putative aminopeptidase. Leucine aminopeptidase RNAs have been shown to be induced in reponse to both water deficient and ABA in tomato (Chao et al., 1999). The two differentially expressed clones isolated from potato, PCR clone 14 and PCR clone 15, are interesting in the sense that both encode proteins that act in signal transduction pathways. Clone 14 is a small GTP-binding protein. Clone 15 is a serine/threonine protein kinase that is homologous to an ABA-inducible kinase in barley aleurone layers (Gomez-Cadenas et al., 1999). The only cytochrome P450 monooxygenase gene (Clone

Figure 4.4. Alignment of the deduced amino acid sequences of cytochrome P450 monooxygenase genes isolated from *Arabidopsis* (Clones 1, 4, 6) and from potato (Clones 12, 13). For comparison, two *Arabidopsis* P450s (accession numbers U61231 and CAA16556) and one *Nicotiana tabacum* P450 (accession number X95342) are included in the alignment. The consensus sequence of the heme-binding domain characteristic of cytochrome P450 monooxygenases is shown above the alignment and is underlined below the sequences. Black boxes designate amino acid sequences that are identical in at least half of the sequences whereas grey boxes designate similar amino acids.

5) that clearly showed differential expression is identical to a member of the CYP73A5 (cinnamate-4-hydroxylase) subfamily. This gene has been shown to be inducible in *Arabidopsis* by many stimuli, including light and wounding (Mizutani et al., 1998). Based on this sequence identity and the known function of the encoded protein, it is unlikely that this is the ABA 8'-hydroxylase gene. PCR Clone 11 encodes a cytochrome P450 monooxygenase as well and shows a slight increase in response to ABA treatment of *Arabidopsis* suspension cultures, with levels decreasing after 24 hours.

Table 4.2. Genes isolated by differential display of *Arabidopsis thaliana* or potato suspension cultures in response to (±)-ABA or trifluoroABA treatment. The genes listed in the table were shown by Northern blotting (Figure 4.5) to increase in response to ABA treatment. The nucleotide sequences on which the putative identifications are based upon are in the Appendix of this thesis.

PCR Clone Number	Gene Identification
2	Unknown
3	Aminopeptidase
5	Cytochrome P450 monooxygenase
14	GTP-binding protein
15	Serine/Threonine Kinase

DISCUSSION

Our results show that modification of the differential display procedure is an effective means of identifying P450 genes. The procedure is less time-consuming than subtractive hybridization, and has the advantage that poly $(A)^+$ RNA need not be used, an important consideration when plant material is limited. The approach used here is similar to that described by Schopfer and Ebel (1998) to amplify cytochrome P450 genes from soybean cultures. In their approach, Schopfer and Ebel (1998) increased the number of individual PCR reactions that had to be run by using a series of non-degenerate primer sets. Each primer within the set represents a potential sequence present in a cytochrome

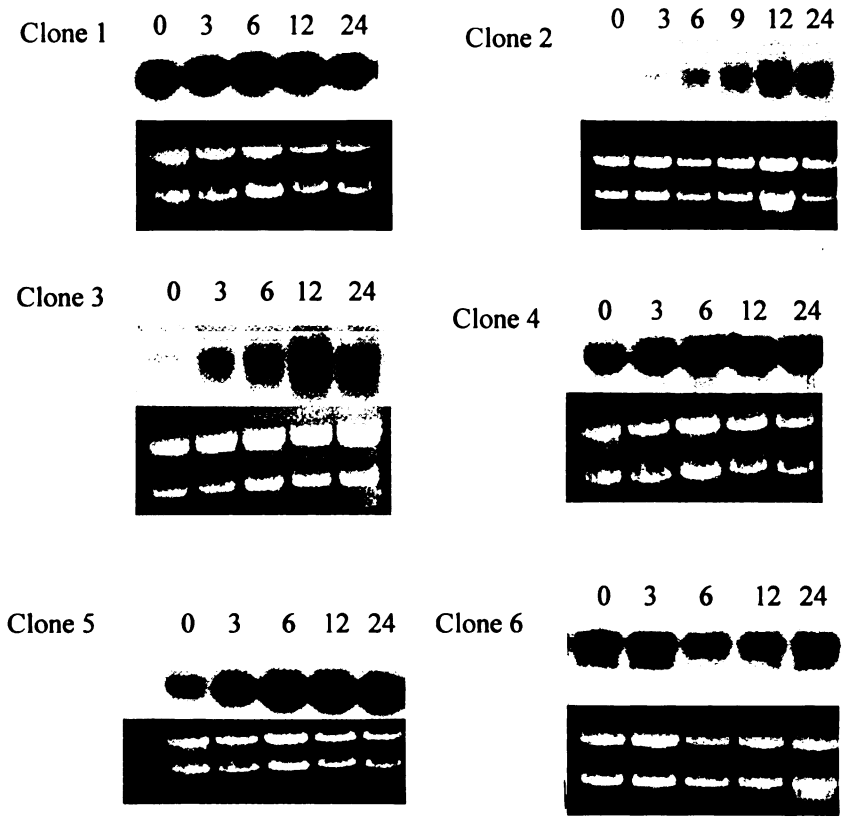

Figure 4.5. Northern blot analysis of genes isolated by differential display. Total RNA was isolated from *Arabidopsis* suspension cell cultures treated for 3, 6, 9, 12, or 24 h with either 50 μM (±)-ABA or with trifluoro-abscisic acid, and from untreated cultures as a control. Potato cultures were treated with ABA in a similar manner as for *Arabidopsis* cultures, and RNA isolated from each time point. DD-PCR was performed using control cultures and 6 h-treated cultures for comparison. Bands that appeared differentially expressed were excised, reamplified, and cloned. The cloned fragments were used as probes on the Northern blots. Under each Northern blot is the respective RNA gel to demonstrate equal loading. The band numbers refer to Table 4.1. Clones 1, 2, 3, 4, 5, 6, 7, and 11 were isolated from *Arabidopsis*. Clones 12, 13, 14 and 15 were isolated from potato. Clones 1, 4, 5, 6, 7, 11, 12, and 13 are putative cytochrome P450 monooxygenase genes.

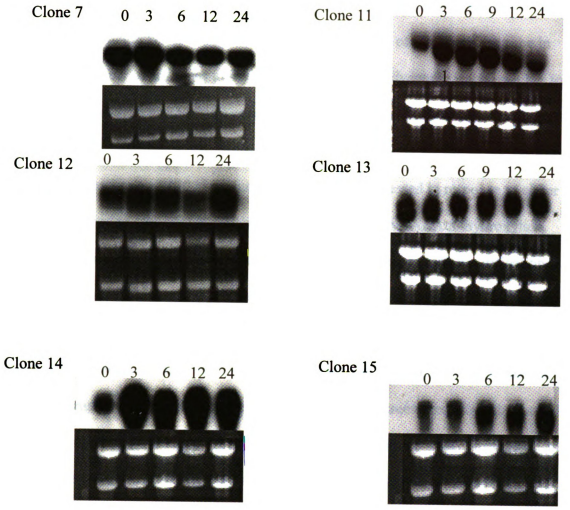

Figure 4.5. (continued)

P450 monooxygenase gene. In contrast, the primer sets used here consisted of a complex mix of species, each of which could potentially be part of a cytochrome P450 monooxygenase gene. While our approach has the advantage of requiring less time in terms of the number of reactions to be set up, a potential disadvantage is the inability to amplify all of the cytochrome P450 genes since the superfamily is known to be diverse in sequence.

Any cytochrome P450 gene that is differentially expressed in response to either ABA treatment or to rehydration following wilting is a putative ABA 8'-hydroxylase. This approach is therefore more rapid, at least initially, than biochemical purification. Ultimately, confirmation of function would require heterologous expression and testing of the enzymatic activity of the recombinant protein. The differential display approach has the obvious disadvantage that some candidates are likely missed. To avoid purifying and expressing all of the genes, a strategy would be to first screen these for substrate specificity in terms of induction. Since ABA 8'-hydroxylase induces its own catabolism and it only catabolizes (+)-ABA (Cutler et al., 1997), *trans*-ABA should be ineffective as inducing the message. Once this sorting limits the number of putative 8'-hydroxylase clones, then yeast expression systems may be employed to determine whether the genes identified encode proteins with the expected function.

An alternative approach being utilized by Cutler's group to identify ABA 8'-hydroxylase is the use of an ABA analog, methylene abscisic acid (Abrams et al., 1997). This substrate analog binds irreversibly to the hydroxylase, and hence, if radiolabeled, would allow isolation of the protein in the form of a radioactive complex that could be visualized on polypeptide gels. Once the protein were identified, the gene would then

need to be obtained, if for example, one wishes to make transgenic plants.

Mutant screening may also be an effective way to identify the ABA 8'-hydroxylase gene. However, screening on the basis of phenotype alone may be insufficient. For example, if one were to screen for mutants that exhibited delayed wilting in response to water stress, various classes of mutants could be isolated that have no involvement in ABA metabolism. It may be necessary to employ a 'brute-force' screen in which one assayed individual plants for ability to make PA.

Given the rate at which the *Arabidopsis* genome is being sequenced, and the existence of various T-DNA and transposon-tagged lines of *Arabidopsis*, it is likely that the ABA 8'-hydroxylase sequence is already available, but that it has yet to have a function assigned to it. To assign function to the numerous cytochrome P450 genes in the database, Winkler et al. (1998) are amplifying various T-DNA tagged cytochrome P450 monooxygenase genes and monitoring the phenotype associated with each insertion. Thus far, numerous genes have been found to have phenotypic effects such as dwarfism, but the wilting characteristics of the plants may not necessarily have been studied. It is interesting to note that Foster and Chua (1998) screened for mutants that are deregulated in ABA gene expression. This screen could potentially yield the ABA 8'-hydroxylase gene, as the inability to metabolize ABA would essentially have the same effect in causing a prolonged activation of genes that would normally show a transient increase to ABA. It will be interesting to see whether any of the deregulated mutants have a genetic defect in a cytochrome P450 monooxygenase.

In order to control ABA levels in plants, the cytochrome P450 monooxygenase gene will need to be identified and characterized. The potential uses of such a gene

include the control of dormancy in crops such as wheat and canola, improved stress tolerance, and possibly better control of growth rate. The differential display approach described in this chapter could potentially be continued, and used successfully to clone ABA 8'-hydroxylase.

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Chapter 5

General Discussion and Prospects

I. Ethylene Biosynthesis

The pathway of ethylene biosynthesis in higher plants has been extensively characterized. The biochemical and molecular details of the enzymes in the pathway, the effect of feedback regulation of ethylene biosynthesis, and the signal transduction pathway leading to ethylene action have all been studied (Johnson and Ecker, 1998). However, the question of the evolutionary origin of the pathway has not been rigorously addressed. Genes encoding enzymes within a biochemical pathway can be obtained by horizontal gene transfer, and new functions derived by gene duplication followed by mutation. Some examples of these are often seen in amino acid biosynthetic pathways (Parsot, 1986; Belfaiza et al., 1986). In terms of the ethylene biosynthetic pathway, ACC synthase, a pyridoxal-phosphate dependent enzyme, may be derived from related aminotransferases that use various amino acids as their substrates. ACC oxidase may be derived from a dioxygenase that incorporates oxygen into the substrate.

For addressing the question of derivation of a biosynthetic pathway, comparisons with other organisms are necessary. Ethylene is produced in fungi, algae, mosses and ferns. A role for ethylene in fungi and green algae has not been established. In ferns and mosses, ethylene may play a role in the transition from the gametophytic to sporophytic phase (Kwa et al., 1995), and in growth in response to submergence (Cookson and Osborne, 1976; Chapter 2, this thesis). Higher plants use ethylene for a variety of roles. Consideration of these roles is important because with the acquisition of new roles, a change in the biosynthetic pathway may have occurred. Further, the transition of plants

from water to land entailed a variety of changes in their morphology and presumably such changes required biochemical modifications. Could one such change have been the derivation of an ethylene biosynthetic pathway that could be more highly regulated than that of lower plants? The difficulty in addressing such a question is that having such a simple chemical structure, ethylene could potentially be derived from numerous chemical precursors. This is supported by initial studies of ethylene biosynthesis in which it was found that linolenic acid could serve as a precursor *in vitro* (Lieberman, 1978).

It is intriguing to speculate that the presence of ACC synthase in ferns could represent the initial evolution of a higher plant ethylene biosynthetic pathway. As discussed in Chapter 2, ACC oxidase is apparently absent in the ferns. It may be that ACC has a role unrelated to ethylene biosynthesis. As discussed in Chapter 2, fungi use α-ketoglutarate as a precursor in ethylene production, and the enzyme that catalyzes this reaction is a dioxygenase (Fukada et al., 1993). In higher plants, ACC oxidase is related to dioxygenases but unlike other dioxygenases, it does not use α-ketoglutarate as a cofactor (Prescott, 1993). The parallels between these two pathways for ethylene production are apparent, but the significance is unknown. Osborne et al. (1996) attempted to use several radioactive compounds as precursors to ethylene, including α-ketoglutarate, but failed to produce radiolabeled ethylene in ferns.

Radiolabeling studies are often difficult to interpret due to dilution effects that may arise from large endogenous pool sizes of the compound. Further, compartmentalization is a frequently encountered problem (Oaks and Bidwell, 1970). It is possible, for example, that in a two-step biosynthetic pathway, one step may occur within an organelle, distinct from the site where the initial precursor is present. In this

way, radiolabel in the initial compound may be either diluted prior to its incorporation into the final product or inaccessible to the enzymes that would utilize it as substrate. It is, therefore, impossible to discount a substance as a precursor on the basis of radiolabeling studies alone. It is possible, for example, that ACC oxidase is indeed present in ferns, but that it is present in a compartment such as a vacuole. It should be noted that results presented in various reports localize ACC oxidase in higher plants in the vacuole as well (Reinhardt et al., 1994), although in this case, radiolabeled methionine gives rise to radiolabeled ethylene.

To address the question of ethylene biosynthesis in the ferns further, one would first have to determine how the pathway is regulated. As alluded to previously, this is important because presumably as the functions attributed to ethylene became more diverse over evolutionary time, very precise regulation and modulation of its biosynthesis became necessary. This question was mentioned in relation to the submergence experiments described in Chapter 2. Finding conditions under which there is an increase in production of ethylene would be useful in the characterization of any enzymes involved in the process. A useful technique to isolate the full-length cDNA for the *Marsilea* ACC synthase gene would be 3' and 5'-RACE. The full-length gene could then be used as a probe on Southern blots, which have thus far been problematic. It is known that some genes cannot be detected on Southern blots, perhaps due to a large number of introns. Northern blots could also be performed in tissues such as the expanding rachis (i.e. after submergence) to see whether message levels for the ACC synthase gene correlate with increases in ethylene production. To find the ethylene biosynthetic enzyme, one could try to use degenerate primers designed against ACC oxidase under

low stringency conditions in RT-PCR, or on genomic DNA, to assess whether a similar gene exists in ferns.

II. Abscisic Acid

Abscisic acid has been traditionally thought of as a stress hormone with levels increasing in response to factors that ultimately cause dehydration (Koornneef et al., 1998). A role for ABA in development has really only been addressed in seed germination and in developing embryos. Evidence for the role of ABA in seed germination and in dormancy is provided by the viviparous mutants whose deficiencies in ABA levels correlate with premature germination (McCarty, 1995). In developing embryos, ABA prevents germination (McCarty, 1995). The mechanism that leads to an increase in ABA has only been established in the case of physiological changes, such as wilting. For this reason, ABA biosynthesis was addressed in fruit to determine whether conserved regulatory mechanisms exist in both physiological and developmental conditions.

As discussed in Chapter 3, ABA biosynthesis in avocado fruit is controlled at the level of xanthophyll cleavage. The implications of the work described in Chapter 3 can be summarized as follows: 9-*cis*-epoxycarotenoid cleavage enzymes exist as members of a multi-gene family. However, not all genes with sequence similarity to 9-*cis*-epoxycarotenoid dioxygenase genes necessarily play a role in ABA biosynthesis, because the localization of some of the 9-*cis*-epoxycarotenoid dioxygenases is not restricted to the thylakoid membrane.

To study the role of the 9-*cis*-epoxycarotenoid enzymes, the use of a system such as tomato may be feasible. It will be interesting to establish whether the expression of

two highly related genes, such as *PaNCED1* and *PaNCED3* described in Chapter 3, is specific to fruit, or whether multiple enzymes with the same function exist in other organs as well. As discussed in Chapter 3, *PaNCED1* and *PaNCED2* are expressed in leaves, but these two genes are not highly related and likely carry out different functions. If two highly related genes are found to be expressed in other organs as well, it will be interesting to determine whether the different enzymes have different localizations (i.e. outer membrane and thylakoid), and whether they carry out the same function.

To determine the function that the outer membrane NCED enzymes described in Chapter 3 play in *in vivo* ABA biosynthesis, it may be useful to determine the orientation within the chloroplast envelope. Use of a tagged-construct coupled with protease protection studies may indicate whether the enzyme spans both the inner and outer chloroplast membranes. It is known that aldehyde oxidase is present in the cytoplasm (Sekimoto et al., 1998), hence it is conceivable that the orientation of the enzyme in the membrane may reflect this role in that the product formed (xanthoxin) may be released on the cytosolic side of the envelope. Also, since the outer membrane of the chloroplast has a different carotenoid composition compared to the thylakoid membrane (Siefermann-Harms et al., 1978), enzymes that localize to the outer membrane may differ in their *in vivo* substrate preference. According to the carotenoid data presented in Chapter 3, both neoxanthin and the two violaxanthin isomers decrease as the fruit ripens, with neoxanthin decreasing to a greater extent. Thus, despite the *in vitro* data that showed that both PaNCED1 and PaNCED3 had a preference for violaxanthin as a substrate, *in vivo,* it would appear that neoxanthin may be the substrate of both enzymes. Cross-linking studies with purified membranes may be useful to determine which

pigment-protein complexes each of the enzymes is associated with. The carotenoid complexes can be isolated, and losses of specific carotenoids in response to the addition of purified enzymes can be determined, as an indication of *in vivo* substrate preference.

Delineation of the promoter regions of *PaNCED1* and *PaNCED3* may also be useful in terms of the isolation of tissue-specific *trans*-acting factors that up-regulate these genes. In addition, fusion of the promoter region from the two genes to reporter genes may reveal whether the promoter directs expression in a tissue-specific fashion and if so, what specific sequences are present to direct the differential expression in different organs and in response to different stimuli.

The outstanding question in ABA biosynthesis is the isolation of the ABA 8'-hydroxylase gene. Approaches discussed in Chapter 4 are useful starting points toward identifying the enzyme. Once isolated, numerous studies can be undertaken with this enzyme, both as an example of a substrate-regulated cytochrome P450 monooxygenase, and as one of the first plant cytochrome P450s to be discovered with a role in hormone catabolism versus hormone biosynthesis. As both the biosynthesis and catabolism are important in governing ABA levels, this thesis attempts to present experimental approaches for addressing both of these processes. Ultimately, understanding the interplay between the enzymes involved in both catabolism and metabolism will be necessary to regulate ABA levels in plants.

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APPENDIX

Nucleotide sequences of the genes cloned in Chapter 4 of this thesis. The clone number matches that given in Table 4.1. The putative identification of the genes, as based on sequence homology, is indicated below the sequence.

Clone 1
GCTGCCGTTCAGGGCGGGGGGAGGATTTGTGCGGCGATAAACATGGCTGAGA
GACTTGTTCTGTTCAACATTGCTTCACTTCTTCACTCTTTTGATTGGAAAGCTC
CTCAAGGACAGAAGTTTGAGGTTGAAGAGAAGTTTGGTCTTGTTCTTAAGTTG
AAGTCTCCACTTGTGGCTATTCCTGTTCCAAGGTTGTCCGATCCAAAACTCTA
CACAGCTTAAGTAAGAAGGATGTTGAATTGAGTTTTCTTGTTTAAAGTTTACT
TTTCTTTATCTGTGTCTGGAGTTTTGATGCTTTGTATAAAAGATGTGTCAAGTT
ACATTTTTGATAACATTTAATATAACAAAGTTTTACTTAAAAAAAAA

The highest match from a Blastx search of this sequence was: CAA16554 (ALO21635) - *Arabidopsis thaliana* cytochrome P450 monooxygenase, Poisson probability = 2e-31

Clone 2
GGCCAGAAGAGTCTTTAAAGGCTTTTTTTCATTGTTCGCATAACAAAAAAGTG
AAACAGAATAGCAAAAGAAGAAAAGAGATACACAAGACTCAGTGTGTCATT
ATTATGATCGGAAATGAGTTTTTCACTCAAGCTCCATTGTTGACAATAAAAAG
AAAACAAATCTTGGTAACTCAGTTACTTTTGAATCCCTTAAGAACATAAGCTC
AAAGTTGACTTCAAACTTCAGGTGTGTCTGACCGCAAAACAGGTTCTGGGGC
TTCCATCAGCCCATTGGAGAAGAGAGAAGTGGAACCTTCTCGCG

A Blastx search of this sequence produced no significant matches.

Clone 3
TGTAAACGACTCACTATAGGCCGANTGGGCCCCGACGTCGCATGCTNCNCCC
CCCCTGGCGGCCNGCGGGAATCCGTTCTGATGCCCGTTCTGGGCCGGGGAGC
CGGTACGATGAAACCCGTCAAGGTCTAGCCAAGGCACAATTGGAAATGATAA
TGTCTGCTAATGGGCTGTCTGAGAATGTCTTTGAGATTGCCTCCAAGAGTTTG
GCTGCTTGAGAGACACGTTAAGAATCCTTTTAAAGACACTTTACTCTGAACTA
TTTACCTACTGAAATTAGTAGTGGGATATGTTAAAATGATCACAGATTGTTTT
GCCTCTGCTTATGAGCTTTACTATTACAATAAACGTTTGCATTATATATTACG
GCAATGTTATTTCTCAAAAAAAAAAA

The highest match resulting from a Blastx search of this sequence was: spP37893 *Caulobacter cresentus* aminopeptidase N, Poisson probability = 0.23

Clone 4
CGAATTGGGCCCCGACGTCGCTGCTCCCCCGCCGCCTGGCGGCCGCGGGAAT
TCGATTAGATGCCCTTCGGGGGGGGGGAGGAGGATTTGTCCCAGGGATTGGG
CTAGCGATGCTGCATCTGGAGTACTATGTGGCGAATATGGTGAGGGAGTTTG
ACTGGAAGGAGGTCCAAGGTCATGAAGTGGACTTGACGGAGAAGCTCGAGTT

TACCGTAGTCATGAAGCATCCGCTTAAGGCCCTTGCTGTTCCTAGGAGAAGTC
ACTAGTTGTCTACTTCTTAAACTTCAGTTTAGTATTCACCAAAGACTAGGTGA
TGTATGAAGAATTGAAGTTATGGGCATTTTGTTAGCTGAAGTCAACTATGGA
ACTATCTTTTTTATTAAAGTACATTTTGTACCTTGGAATAAATGATAGAAACA
ACAATCCTTTTATTTTGTAAAAAAAAAAA

The highest match from a Blastx search of this sequence was: gi1432145 (U61231)
Arabidopsis thaliana cytochrome P450 monooxygenase, Poisson probability = 4.3e-28

Clone 5
CCGTTTGGTGTTGGACGTAGAAGCTGTCCCGGGATTATATTGGCATTACCTAT
TTTGGGGATCACCATTGGTAGGATGGTCCAGAACTTCGAGCTTCTTCCTCCTC
CAGGACAGTCTAAAGTGGATACTAGTGAGAAAGGTGGACAATTCAGCTTGCA
CATCCTTAACCACTCCATAATCGTTATGAAACCAAGGAACTGTTAAACTTTCT
GCACAAAAAAAAGGATGAAGATGACTTTATAAATGTTTGTGAAATCTGTTGAA
ATATTCCCTTGTTTTGCTTTTGTGAGATGTTTTTGTGTAAAATGTCTTTAAATG
GTTGTTCTACGATTGCAATAATAATTAGTGGTGCTCATTCTTAAAAAAAAAAA
AAA

The highest match from a Blastx search of this sequence was: GI:4096693 (U37325)
Arabidopsis thaliana cytochrome P450 monooxygenase, Poisson probability = 3e-24

Clone 6
CCGTTTGGGGCGGGGAGGCGGATTTGTGCGGCGATAAACATGGCTGAGAGAC
TTGTTCTGTTCAACATTGCTTCACTTCTTCACTCTTTTGATTGGAAAGCTCCTC
AAGGACAGAAGTTTGAGGTTGAAGAGAAGTTTGGTCTTGTTCTTAAGTTGAA
GTCTCCACTTGTGGCTATTCCTGTTCCAAGGTTGTCCGATCCAAAACTCTACA
CAGCTTAAGTAAGAAGGATGTTGAATTGAGTTTTCTTGTTTAAAGTTTACTTT
TCTTTATCTGTGTCTGGAGTTTTGATGCTTTGTATAAAAGATGTGTCAAGTTAC
ATTTTTGATAACATTTAATATAACAAAGTTTTACTTGCAAAAAAAAAAAAAAA
AAA

The highest match from a Blastx search of this seequence was: CAA16554 (ALO21645)
Arabidopsis thaliana cytochrome P450 monooxygenase, Poisson probability = 2e-35

Clone 7
CCTTTCGGGTCGGGCCGGAGAATGTGCCCGGCCATGCATCTTGGGATTGCAA
TGGTAGAGATACCTTTCGCTAACCTTCTCTACAAATTTGACTGGAGTCTACCT
AAAGGGATTAAACCAGAGGATATAAAGATGGACGTCATGACTGGACTCGCTA
TGCACAAGAAAGAACACCTCGTTCTTGCACCAACGAAACACATCTGATGCTA
TATATATCATTAGGACGTTTCTGCTGGTAGATATGGCGTGACCAATGGTTATT
TTTCATTGCAATATCCCTTTTTGTTTTAATGAGTACTATGTTCTCATTTTAACG
AATAAAAATGTATCAGTGCTCTTGTTTTTGGACTAGAAAAAAAAAA

The highest match from a Blastx search of this sequence was: BAA28531 (D78598)
Arabidopsis thaliana cytochrome P450 monooxygenase, Poisson probability= 6e-35

Clone 8
TACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCNCNNCCGCC
ATGGCGGCCGCGGGAATTCGATTCAGTTGCCGTTTCTGGGCCGGGGGGCGTA
TAGGATGGGTTTGAGGGTTTTAAATGGCCTAGAATGAATACTTTACTTAAGA
GTCCCAGGCTCATTCTTCAGTTAAATGCCTTACTTTCAGGTTTGCTTTCTCTTA
TCAAATAAGGGAAATACTTGCTATCCGAAAAGTGGTTCATTCATCTTAGATG
ACAAAGGAATCATCTTTGAACAGTATTGTAAACAGATATCCTGATAATGATA
ATTTTCGTCTGC

The highest match from a Blastx search of this sequence was: g322598 *Arabidopsis thaliana* st12p, Poisson probability= 28e-12

Clone 9
TAGCTGCCGTTCGGGGGGGGGCGGCGGGGATGACATATATATCGTATTCAAA
CATCTGTGCGAATATCGTGAGGAACTGTCTCAAGGAGCCACACAAAGCTGAA
GCTCTTACTCGCGAGAAGGTTCACTTCTCTCTCTCCAAATGGGCTGATGGAAG
GCCCCAGAAACCTGTTTTGCGGTCAGACACACCTGAAGTTTGAAGTCAACTTT
GAGCTTATGTTCTTAAGGGATTCAAAAGTAACTGGGTTACCAAGATTTGTTTT
CTTTTTATTGTCAACAATGGAGCTTGAGTGAAAAACTCATTTCCGATCATAAT
AATGACACACTGAGTCTTGTGTATCTCTTTTCTTCTTTTGCTATTCTGTTTCAC
TTTTTGTATGCGAACAATGAAAAAAGCCTTAAAGACTCTCTGGCCAAAAAAA
AAAAAA

The highest match resulting from a Blastx search of the sequence was: spQ96253 *Arabidopsis thaliana* ATP synthase episilon chain, Poisson probability= 3e-28
Clone 10
TAGCTGCCGTTCGGGTGGGGGGGGCGATCAGGAGTGGAAGTTCTACTGTTAG
TGTGTTTTCATTGCCGCCTCTACAGTTAAATGGCTTAGATGAGACATGGAAGA
AGATTCCATTGTTGGAGCAAGAAGCCAAATAGAGAGTGAGACAGAGGCACA
CTCGCTCTATTCCACTTTTTTATACAATAGAAGAAGCTAACAGATTAAACAAG
ACATTATCATCAATGTACACAAAAAGGCAGAAGAAACTATAGGATCATGTTT
CTGGATATCATCTTTGGTCGGTCATAAGTCCTTAGGTTACACAAAACTGCAAT
GTAACTTTGTTTCTGTGTTGGGGAAAAAAAAAAAAAAAA

A Blastx search of this sequence produced no significant matches

Clone 11
ACCATGGCTCCACTTCTNCACTCTTTTGATTGGGAAAGCCNCCTCAGGGACAA
AAGTTTGGAGGTTGGAAGAGAAGTTTGGTCTTGTTCTTAAGTTGAAGTCTCCC
ACTTGTGGCTATTCCCTGTTCCCAAGGTTGTCCGATCCAAAACTCTATACAGC
TTAAGTAAGTAAGAAGGAAATTTGGTTTGAGCTTATGAGAGGAAGTTGAATT
GAGTTTTCTTGTTTAAAGTTTATTTTTCTTTATTCGTGTTTGGAGTTTGATGCTT
TGTAAAAAAAGATGTGTCAAGTTAAATTTAATTNTCTTATATATAATAAAATA
AAGTTTTAGTCACAATAAAAAAAAAAA

The highest match resulting from a Blastx search of this sequence was: CAA16556 (ALO21635), *Arabidopsis thaliana* cytochrome P450 monooxygenase, Poisson probability= 1e-06

Clone 12
CCGTTTGGGCTGGGGAGGCGGAGGTGCCCGGGGTATAGCCTAGGTATGAAGG
TTGTTCGAACAACAATGGCTAACTTGTTACATGGATTCAATTGGAAATTAGCA
GGAGATACGAGGCCAGAAGATATAAGCATGGAGGAGATTTATGGATTAACT
ACACACCCAAATAAGCCTATATCTGTGATTATGGAACCTAGACTTCCCTTCCA
TCTTTATTAGAACTAGCTATAGTACTTTGTTTTGGTCTTGCCAATATAAGTTTT
ATGCTAGCATTAAGTTAAAATAAAATTCCACTTTTTGACGAAAAAAAAAAA

The highest match resulting from a Blastx search of this sequnce was: PID e220214 (X95342) *Nicotiana tabacum* cytochrome P450 monooxygenase, Poisson probability= 1.3e-25

Clone 13
CTACACACCCAAATAAGCCTATATCTGTGATTATGGAACCTAGACTTCCCTTC
CATCTTTATTAGAACTAGCTATAGTACTTTGTTTTGGTCTTGCCAATATANGTT
TTATGCTAGCATTAAGCTAAAATAAAATTCCACTTTTTGACGAAAANAAAAA
AGCTTAATCACTAGTGAATTCGCGGTCGCTGCAGNTCGACCATATGGNAGAG
CTCCCAACGCGNTGGATGCATAGCTTGAGTATTCTATAGTGCCACCTAAATAG
CTTGGCGTAATCATGGTCATAGCTGCTTCCTGTGTGAAATTGCTATCCGCTCA
CANTTCCACACAACATACGAGCCG

The highest match resulting from a Blastx search of this sequence was: CAA64635 *Nicotiana tabacum* cytochrome P450 monooxygenase, Poisson probability= 5e-12

Clone 14
CATACATAGAGTGCAGCTCAAAAGCACAAATGAACGTAAAAGCGTGTTTG
ATGAAGCGATCAAAGTAGTTTTACATCCTCCTCAAAGACTAAGAAGCGAA
GAGAAAGAT CGGTTTATGCCATGTTCTTT GACTAGTGTACTCAAGTCTCTG
GCTTTGGT TTGTTTTACATCTTTTTTAG TGTGTTTCTTCTATGAAGGACCTC
TTGCAGATTTTAGTATGATTAGCTATC TTCGTTATAAAAGTTTGATCTTTTG
TAGTAGAG

The highest match resulting from a Blastx search of this sequence was: BAA84494 (ABO2930) *Oryza sativa* small GTP binding protein, Poisson probability=5e-5

Clone 15
TGCATTTAGCTGACGGNGAAACTTTAGCCAAGGGTGAGCTGATTTTCGNGCG
ANTAAACCTGAAGGTTCTAGACCCGCGTGGATCCAATCACCTAACAGGAAGC
CTTAACTGTTGGCTCATGAACTCGAACAAAGCGTGGGGTCTTCCTCAATCCAT
GTCTTTCGATAAAAATGAGGGGGCTTACTTGGATCGTGAAGGGACTTTGGAG
GTAGAGATTGAATGCGAAATTAAAAACTCCCATAAAAACCATCCCTTCTTTTA
GGATATCAACCATGTCAGGTCTACTACTCTAGAGTCTGGGACTACTCTGTTTT

AAGCGTAAAAACTGAATGTGAAGTTAACGCTCTATCTATGTTTAGGATATCTA
TGTATCTACCATGCCATGTCTACTACTCTATGACTTACCTACTCTGGTTTTAGG
ATATCTAAATATCTACCATGCCAAAAAAAAAAA
The highest match resulting from a Blastx search of this sequence was: AAD26871.1 (AC007230) *Arabidopsis thaliana* T23K8 (serine/threonine kinase), Poisson probability=2e-45

Clone 16 = identical to clone 13

Clone 17
CACTATAGGGGCAATTGGGCCCGACGTCGCAATGCTNCCNCCGCCATGGCGG
CCGCGGGAATTCGATTCTGCTGCCGTTCTCGGGTGGGGCGGCGAGAGCGGTA
CCAAATCGAGGCAAACTCTGAATACTAGTTTACTAGATATGACCTCAAAATA
ACTGGGGTCAAGGTCGGCCAGTGAGAGACGGTGGGGGATAAGCTTCATCGTC
GAGTCGAGAGGGGAAACAGCCCGGATCACCAGCTAAGGCCCCTAAATGACC
GCTCAGTGATAAAGGAGGTAGGGGTGCAGAGACAGCCAGGAGGTTTGCCTA
GAAGCCAAAAAAAAAA

A Blastx search of this sequence produced no significant matches

Clone 18
TAATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCC
GCCATGGCGCTCNGNGNGAATTCGATTTAGCTGCCGTTCGGGTGGGGGGGGC
GATCAGGAGTGGAAGTTCTACTGTTAGTGTGTTTTCATTGCCGCCTCTACAGT
TAAATGGCTTAGATGAGACATGGAAGAAGATTCCATTGTTGGAGCAAGAAGC
CAAATAGAGAGTGAGACAGAGGCACACTCGCTCTATTCCACTTTTTTATACA
ATAGAAGAAGCTAACAGATTAAACAAGACATTATCATCAATGTACACAAAAA
GGCAGAAGAAACTATAGGATCATGTTTCTGGGATATCATCTTTGGTCGGTCAT
AAGTCCTTAGGTTACACAAAACTGCAATGTAACTTTGTTTCTGTGGTTGGGGA
AAAAAAAAAAAAAAA

A Blastx search of this sequence produced no significant matches.