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presented by

Steven H. Schwartz

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of the requirements for

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Jan Zeevaart
Major professor

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THE REGULATION OF OSMOTIC STRESS RESPONSES

By

Steven H. Schwartz

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

PHD

Botany and Plant Pathology

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ABSTRACT

By

Steven H.Schwartz

Osmotic stress may result from drought, high salinity, or freezing temperatures. All three stresses cause a reduction in water potential, efflux of water from cells, and a loss of turgor. Plants have evolved a variety of responses to cope with osmotic stress. The regulation of these responses in higher plants and in a cyanobacterial model system are the focus of this disseration.

Salt-induced genes were identified in the cyanobacterium, *Anabaena* sp. PCC 7120, by promoter trapping with a Tn5::*luxAB* construct. Second-site mutagenesis was used to identify regulatory components necessary for the salt-induction of this gene. One mutant which displays reduced luciferase activity during salt stress has an insertion in an ORF with sequence similarity to response regulators from two-component regulatory systems. The mutation was reconstructed with an interposon based vector and shown to have the same phenotype.

In higher plants, the hormone abscisic acid (ABA) regulates many responses to osmotic stress. ABA is formed by the oxidative cleavage of an epoxy-carotenoid. This is the first committed reaction in ABA biosynthesis. The reaction is of general interest, because the synthesis of other apocarotenoids, such as vitamin A in animals, may occur by a similar mechanism. A new ABA-deficient mutant of maize has been identified and the corresponding gene, *VP14*, has been cloned. The recombinant VP14 protein catalyzes the cleavage of 9-*cis*-epoxy-carotenoids to form C_{25} apo-aldehydes and xanthoxin, a precursor

of ABA in higher plants.

Following the cleavage reaction, xanthoxin is oxidized to ABA in two steps. The *aba2* and *aba3* mutants of Arabidopsis, are impaired in these later steps. The *aba2* mutant is blocked in the conversion of xanthoxin to ABA-aldehyde and *aba3* is impaired in the conversion of ABA-aldehyde to ABA. Extracts from the *aba3* mutant also lack several additional activities which require a molybdenum cofactor (Moco). Nitrate reductase utilizes a Moco but its activity is unaffected in extracts from *aba3* plants. Further characterization of the *aba3* mutant indicates that it is impaired in the introduction of sulfur into the Moco.

ACKNOWLEDGMENTS

I would like to acknowledge and thank the following people for their contribution to the work presented in this dissertation. Karen Léon-Kloosterziel and Maarten Koornneef provided the *aba2* and *aba3* mutants of Arabidopsis. Don McCarty and Bao-Cai Tan identified the *vp14* mutant in maize and cloned the corresponding gene. Mike Thomashow, Peter Wolk, and Ken Poff served on my committee. I would also like to thank Jan Zeevaart for his guidance during my graduate studies.

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INTRODUCTION

Drought and salinization are among the most serious problems facing food production in the world. In the U.S., 25 % of the land has been classified as arid and 40 % of crop losses can be attributed to drought (Boyer, 1982). In addition, current irrigation practices have led to the rapid salinization of agricultural lands. In recent years, plant breeding for increased drought tolerance has provided some improvements (Yeo, 1994). Some major crops, however, lack the diversity in their gene pool for adaptation to arid environments. In these instances, genetic engineering may allow proven strategies of drought and salinity tolerance to be introduced into drought-sensitive plants (Bohnert and Jensen, 1996). Therefore, an understanding of drought tolerance strategies and their regulation should be beneficial for crop improvement. The work described in the following chapters is intended to provide some insight into the regulation of osmotic stress responses in higher plants and in the cyanobacterium, *Anabaena* sp. PCC 7120.

Mechanisms of drought stress tolerance.

Osmotic stress may result from drought, high salinity, or freezing temperatures. All three stresses cause a reduction in water potential (Ψ), efflux of water from cells, and a loss of turgor. Plants have evolved a variety of mechanisms to survive in environments with low water potentials. Many of these strategies involve complex biochemical, morphological and phenological processes, which allow plants to utilize the available water efficiently. For example, CAM photosynthetic plants temporarily fix carbon at night when transpiration rates are low (Ting, 1985). In environments with predictable dry and wet seasons, some annual plants remain dormant during the dry season and undergo rapid growth and reproduction during the wet season (Aronson et al., 1992). Many plants increase the root to shoot ratio during water stress to elevate water uptake (Wu et al., 1994).

Dessication tolerant plants such as the resurrection plant, *Craterostigma plantagineum*, are able to undergo severe dehydration and remain viable. A number of genes are induced during osmotic stress in higher plants (Ingrams and Bartels, 1996). The induction of specific transcripts during osmotic stress suggests that these genes have a function in dessication tolerance. Engineering desiccation tolerance should be easier than altering the complex morphological and developmental processes mentioned above. The function of genes encoding the enzymes for the synthesis of compatible solutes is well established (McCue and Hanson, 1990; Tarcynski et al., 1993; Bohnert and Jensen, 1996). Many genes have been identified by differential screening techniques, but their function has not yet been determined. Functions have been hypothesized for several stress inducible genes, based on sequence similarity (summarized in Ingrams and Bartels, 1996). Late embryogenesis abundant genes (*Lea*), first identified in dessicated cotton seeds (Baker et al., 1988), are ubiquitous in plants and are usually the most abundant osmotically induced genes (Ingram and Bartels, 1996). Secondary structure predictions for LEA proteins indicate randomly coiled motifs, which may serve a role in binding of water (Baker et al.,

1988) or hydration of proteins (McCubbin et al., 1985). However, there is little experimental evidence to support either hypothesis.

Using heterologous probes, potential homologs of osmotically induced genes in plants were tentatively identified in the cyanobacterium *Anabaena* sp. PCC 7120 (Curry and Walker-Simmons, 1993; Lammers and Close, 1993). These genes are also induced by osmotic stress in *Anabaena*. Following endosymbiosis, a number of stress responsive genes may have been transferred to the plant nucleus. Cyanobacteria, which are more amenable to genetic manipulation, may serve as a good model system in determining the function of these genes. The *Synechocystis* genome (Kaneko et al., 1996) was searched with the translated sequence of several LEA proteins. The ORFs which shared the highest similarity with the *Lea* genes had Poisson probabilities of .002 and greater. From these results it is uncertain why the *Lea* genes hybridized with osmotically induced genes in cyanobacteria (Curry and Walker-Simmons, 1993; Lammers and Close, 1993). There is, however, a potential homolog of another osmotically induced plant gene, the wheat *esi3* gene (Gulick et al. 1994), in the *Synechocystis* genome (Poisson probability of 3.52×10^{-22}).

Abscisic acid function and biosynthesis.

The plant growth regulator, abscisic acid (ABA) (Figure 1.1), controls a number of physiological processes in plants, such as embryo development, seed germination and stress tolerance (Zeevaart and Creelman, 1988). The various functions attributed to ABA are based upon the effect of its exogenous application and correlations between the endogenous concentrations of ABA and a given process. In more recent years, the

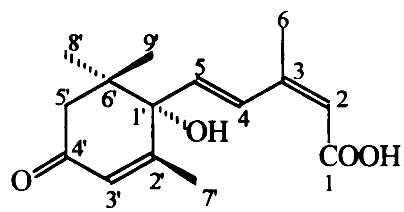


Figure 1.1- The structure of abscisic acid (ABA)

identification and characterization of ABA-deficient has provided definitive evidence for the role of ABA in various processes, such as seed dormancy (Koornneef, 1982).

Physiological responses to ABA may be regulated by changes in distribution, concentration, or sensitivity. A redistribution of ABA in response to osmotic stress (Slovik and Hartung, 1992) may be responsible for rapid changes such as stomatal closure. Physiological changes associated with osmotic stress are also enhanced by increased ABA levels (Zeevaart and Creelman, 1988). Different strains of wheat with distinctive germination kinetics have varying sensitivities to exogenous ABA (Steinbach et al., 1995; Walker-Simmons, 1987). A farnesyl transferase involved in ABA signal transduction (Cutler et al., 1996) may be the molecular basis for this varying sensitivity.

The characteristics of the early steps in ABA biosynthesis have been inferred by carotenoid analysis and $^{18}O_2$ labeling experiments. The carotenoid content in leaves is in great excess relative to the amount of ABA produced and no changes in the carotenoid composition have been associated with elevated ABA biosynthesis. In etiolated tissue, however, the carotenoid concentration is low and a decrease in violaxanthin and neoxanthin has been correlated with an increase in ABA and its catabolites (Li and Walton, 1995). The derivation of ABA from xanthophylls is also supported by $^{18}O_2$ labeling experiments. In the presence of $^{18}O_2$, there is no incorporation of ^{18}O into the 4'-keto or the 1'-hydroxyl of ABA. This data indicates ABA is synthesized from a large precursor pool with oxygen at these positions. ^{18}O labeling does occur at one position in the carboxyl group (Creelman and Zeevaart, 1984), while the other oxygen is derived from water (Creelman et al., 1987). This is consistent with the oxidative cleavage of an epoxy-carotenoid precursor and indicates that *de novo* synthesis of the precursor is not necessary for ABA biosynthesis.

The immediate product of the cleavage reaction, xanthoxin, is rapidly converted to ABA *in vivo* and *in vitro* (Sindhu and Walton, 1987) indicating that the later steps in the pathway are not rate limiting. This conclusion is further supported by the incorporation of ^{18}O in the carboxyl group of ABA. If the aldehyde is not oxidized quickly, the oxygen would exchange with water and the label would be lost (Zeevaart et al., 1989).

The experiments discussed above indicate that the early steps in ABA biosynthesis (the formation of the epoxy-carotenoid precursor) is not rate limiting and the enzymes catalyzing the later steps of ABA biosynthesis (the two step oxidation of xanthoxin to ABA) are constitutively expressed. Therefore, the cleavage reaction in ABA biosynthesis appears to be the rate limiting step.

The inhibition of stress-induced ABA biosynthesis by actinomycin D suggests that the pathway is transcriptionally regulated (Guerrero and Mullet, 1986), perhaps by increased expression of the cleavage enzyme. The compartmentation of carotenoids in the chloroplast envelopes and the thylakoid membranes, however, complicates any consideration of the role that substrate availability has in regulating this reaction. If the cleavage reaction occurs in the envelopes, where only a small percentage of the total carotenoids are contained, this reaction may be substrate limited. There does, however, appear to be a rapid flux of carotenoids between the thylakoid and envelope membranes (Siefermann-Harms et al., 1978).

Altering the expression of the cleavage enzyme is the most reasonable approach for manipulating ABA levels in plants. Because ABA is an endogenous regulator of many drought stress responses, altering its levels could have a dramatic effect on drought tolerance.

REFERENCES

Aronson J, Kigel J, Shmida A, Klein J (1992) Adaptive phenology of desert and Mediterranean populations of annual plants grown with and without water stress. Oecologia **89:** 17-26.

Baker J, Steele C, Dure L III (1988) Sequence and characterization of 6 *Lea* proteins and their genes from cotton. Plant Mol Biol **11:** 277-91

Bohnert HJ, Jensen RG (1996) Strategies for engineering water-stress tolerance in plants.Trends Biotechnol **14:** 89-97

Boyer JS (1982) Plant productivity and the environment. Science **218:** 443-448

Close TJ, Lammers PJ (1993) An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins. Plant Physiol **101:** 773-779

Creelman RA, Gage DA, Stults JT, Zeevaart JAD (1987) Abscisic acid biosynthesis in leaves and roots of *Xanthium strumarium*. Plant Physiol **85:** 726-732

Creelman RA, Zeevaart JAD (1984) Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. Plant Physiol **75:** 166-169

Curry J, Walker-Simmons MK (1993) Sequence analysis of wheat cDNAs for abscisic acid-responsive genes expressed in dehydrated wheat seedlings and the cyanobacterium, *Anabaena*. *In* Plant Responses to Cellular Dehydration During Environmental Stress. Ed. Close T.J., Bray E.A. American Society of Plant Physiologists, Rockville, MD

Cutler S, Ghassemian M, Bonetta D, Cooney S, McCourt P (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. Science **273:** 1239-1240

Guerrero F, Mullet J (1986) Increased abscisic acid biosynthesis during plant dehydration requires transcription. Plant Physiol **80:** 588-591

Gulick PJ, Shen W, An H (1994) ESI3, a stress-induced gene from *Lophopyrum elongatum*. Plant Physiol **104:** 799-800

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S,Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res **3:**185-209

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin-sensitive lines of *Arabidopsis thaliana* (L) Heynh. Theor Appl Genet **61:** 385-393

Li Y, Walton DC (1990) Violaxanthin is an abscisic acid precursor in water-stressed dark-grown bean leaves. Plant Physiol **92:** 551-559

McCubbin WD, Kay CM (1985) Hydrodynamic and optical properties of the wheat Em protein. Can J Biochem **63:** 803-810

McCue KF, Hanson AD (1990) Drought and salt tolerance: towards understanding and application. Trends Biotechnol **8:** 358-362

Siefermann-Harms D, Joyard J, Douce R (1978) Light-induced changes of the carotenoid levels in chloroplast envelopes. Plant Physiol **61:** 530-533

Sindhu RK, Walton DC (1987) The conversion of xanthoxin to abscisic acid by cell-free preparations from bean leaves. Plant Physiol **85:** 916-921

Slovik S, Hartung W (1992) Compartmental distribution and redistribution of abscisic acid in intact leaves. III. Analysis of the stress-signal chain. Planta **187:** 37-47

Steinbach HS, Benech-Arnold RL, Kristof G, Sanchez RA, Marcucci-Poltri S (1995) Physiological basis of pre-harvest sprouting resistance in *Sorghum bicolor* (L.) Moench. ABA levels and sensitivity in developing embryos of sprouting-resistant and -susceptible varieties. J Exper Bot **46:** 701-709

Tarczynski MC, Jensen RG, Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science **259:** 508-510

Ting, IP (1985) Crassulacean acid metabolism. Annu Rev Plant Physiol **36:** 595-622

Walker-Simmons M (1987) ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiol **84:** 61-66

Wu Y, Spollen WG, Sharp RE, Hetherington PR, Fry SC (1994) Root growth maintenance at low water potentials. Increased activity of xyloglucan endotransglycosylase and its possible regulation by abscisic acid. Plant Physiol **106:** 607-615

Yeo AR (1994) Physiological criteria in screening and breeding. Monographs on Theor Appl Genet **21:** 37-59

Chapter 2

Molecular analysis of salt stress responses in *Anabaena* strain PCC 7120

ABSTRACT

Salt-induced genes were identified in the cyanobacterium, *Anabaena* sp. PCC 7120, by promoter trapping with a Tn5::*luxAB* construct. The genomic sequence adjacent to one insertion was nearly identical with the *lti2* gene from *Anabaena variabilis*, previously identified in a differential screen for cold-induced transcripts. Second-site mutagenesis was used to identify regulatory components necessary for the salt-induction of this gene. One mutant which displays reduced luciferase activity during salt stress has an insertion in an ORF with sequence similarity to response regulators from two-component regulatory systems. The mutation was reconstructed with an interposon based vector and shown to have the same phenotype. Further biochemical and physiological characterization indicates that several salt-induced proteins are absent and that the mutant is more sensitive to salt stress.

INTRODUCTION

In cyanobacteria, a number of adaptations to salt stress have been characterized. For example, the extrusion of ions that are detrimental to cellular processes is critical for survival. Several potential H^+/Na^+ antiport systems are present in the *Synechocystis* genome (Kaneko et al., 1996) and there is biochemical evidence for a H^+/Na^+ antiport systems (Packer et al., 1987). A P-ATPase is involved in the efflux of Na^+ from yeast (Haro et al., 1991) and the disruption of genes encoding P-ATPases leads to decreased salt tolerance in *Synechococcus* (Kamamaru et al., 1993).

Compatible solutes decrease the intracellular water potential to reduce the efflux of water and may also substitute for water in stabilizing membranes and proteins. Cyanobacteria accumulate a variety of compatible solutes in response to osmotic stress (Reed and Stewart, 1988; Reed et al., 1984). Uptake systems for compatible solutes occur in a number of prokaryotes, including cyanobacteria (Mikkat et al., 1996).

As many as 100 genes are induced by salt stress in *Anabaena torulosa* (Apte and Haselkorn, 1990). Some salt-induced genes may be involved in the adaptations discussed above; the function of most salt-inducible genes, however, is still unknown. Among these genes there may be homologs of desiccation induced genes in higher plants (Close and Lammers, 1993; Curry and Walker-Simmons, 1993). The function of these genes in plants is also unknown.

The function of salt-inducible genes may be determined by disrupting the individual genes and determining its effect on salt tolerance. Considering the large number of genes induced by salt stress, the disruption of individual genes may not produce a visible phenotype. Regulatory mutants impaired in the salt induction of multiple genes may be more suitable for these studies.

Two-component regulatory systems mediate environmental responses in a wide range of prokaryotes. Most two-component systems consist of a sensor and a response regulator (Gross et al., 1989; Swanson et al., 1994). The sensor is usually a trans-membrane protein which perceives changes in the extracellular environment and auto-phosphorylates a histidine in its cytoplasmic or "transmitter" domain. The phosphate is subsequently transferred to an aspartate in the amino terminus of the cognate response regulator, which may then alter transcription or other cellular processes. Two-component regulatory systems are often identified on the basis of sequence similarity. The cytoplasmic domain of the sensor kinase is highly conserved and the amino terminus of response regulators share 20-30% amino acid identity on average. While the carboxyl terminus of response regulators is not conserved, the secondary structure often has a helix-loop-helix DNA binding motif (Pabo and Sauer, 1992).

In cyanobacteria, several functions have been attributed to two-component regulatory systems (Campbell et al., 1996; Chiang et al., 1992) including the regulation of salt-stress responses in the cyanobacterium, *Synechocystis* (Hagemann et al., 1996). In addition, changes in protein phosphorylation in response to salt stress have been reported (Hagemann et al., 1993). A number of response regulators have been identified in the *Synechocystis* genome sequence (Kaneko et al., 1996), but their function is not yet known.

An insertional mutant impaired in the induction of several salt-induced proteins has been identified in *Anabaena* PCC 7120. At the site of the insertion there is an ORF which shows sequence similarity to a number of response regulators from two-component regulatory systems.

MATERIALS AND METHODS

Culture conditions.

Anabaena sp. PCC 7120 was grown on AA medium (Allen and Arnon, 1955) containing 1% bacto-agar (Difco) and supplemented with 10 mM NO_3^-. Liquid cultures were grown in AA/8 medium plus 5 mM NO_3^- with continuous shaking. All cultures were grown at 30° C under fluorescent lights.

Transformation of *Anabaena*.

Plasmids were introduced into *Anabaena* sp. PCC 7120 by tri-parental matings on membrane with *E. coli* strain J53 (RP-4) (Wolk et al., 1984). A second *E. coli* strain contained the appropriate plasmid with a RK2 origin of transfer for conjugal transfer and pRL528, which contains the methylases for *Ava*I and *Ava*II restriction sites (Elhai and Wolk, 1988).

Identification of salt-induced genes.

Anabaena sp. PCC 7120 was mutagenized with pRL1063a (Wolk et al., 1991). Insertions into salt-induced genes were identified by comparing photonic images luciferase acitivity prior to and following a hyper-osmotic shift. For the hyper-osmotic shift, filters were transferred to medium containing an additional 100 mM NaCl and a second photonic image was taken after five hours.

Cloning genomic DNA adjacent to Tn5 insertions.

DNA was purified from the AB5 mutant according to a published protocol (Cai and Wolk, 1990), digested with *Eco*RI, and ligated in a 100 µL volume. The ligation mixture was precipitated and used to transform *E. coli* DH10B by electroporation. An origin of replication and the neomycin marker allowed for selection of intra-molecular ligation products, which contain the 1063-Tn5 insertion and the adjacent genomic DNA. A genomic fragment at the site of the SA6 insertion was cloned from this mutant in a similar manner. Fragments of genomic DNA cloned in this manner will subsequently be referred to as rescued plasmid.

Measurement of luciferase activity in liquid cultures.

The indicated concentration of NaCl was added to early log phase cultures of the AB5 insertion. The cultures were incubated for 2 hr before luciferase activity was measured. The luciferase substrate was prepared by sonicating 10 µL of n-decyl aldehyde in 5 mL of water with 100 mg of BSA. The luciferase activity was measured in a scintillation counter with 200 µL of the substrate.

Restructuring the AB5 mutant and second-site mutagenesis.

The plasmid, pRL386a (unpublished plasmids from the lab of C.P. Wolk) contains the *luxAB* genes for recombination with the original Tn5-1063 insertion (Figure 2.1). Following transfer of pRL386a to the AB5 strain, the erythromycin (Em) resistance marker allowed for selection of single recombinants. Adjacent to the resistance markers there is portion of the Tn5 IS*50* R and a *sacB* gene which is conditionally lethal in *Anabaena* (Cai and Wolk, 1990). Several single recombinants were grown in liquid culture and plated on medium containing 5% sucrose and 10 µg/mL erythromycin to select for double recombinants lacking the *sacB* gene. Selected double recombinants showed the same

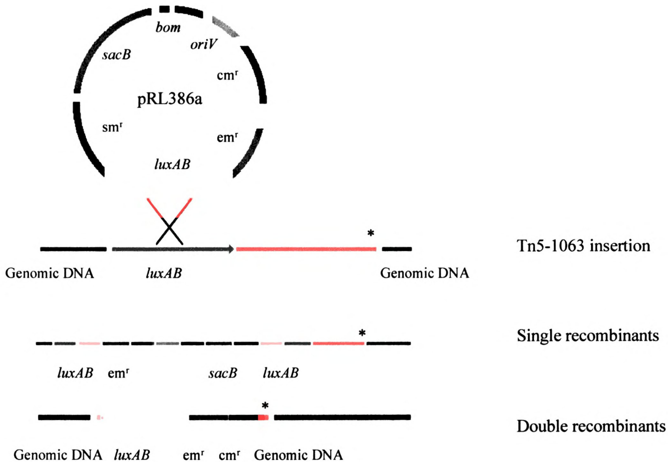


Figure 2.1- Restructuring of Tn5-1063 insertions with pRL386a. The asterisk (*) indicates where the second recombination event occurs.

luciferase induction as the initial insertion. One double recombinant, AB5dr1, was used for second-site mutagenesis with pRL1058 which contains a Tn5 derivative (Wolk et al., 1991). Following mutagenesis, colonies growing on membranes were transferred from AA N medium to medium containing an additional 100 mM NaCl. After five hours, a photonic image of the colonies was taken and superimposed on an analog image to identify colonies with reduced luciferase activity.

Screening for a genomic clone of SA6.

A plasmid rescue from SA6 was used as a template in a random prime labeling reaction (Promega) and used to screen an EMBL3 library (Black and Wolk, 1994). Positive plaques were isolated and phage DNA was prepared from plate lysates (Sambrook et al., 1989) and digested with *Sal*I. A 15 kb fragment of *Anabaena* DNA was isolated and ligated into the *Sal*I site of pRL171 (Elhai and Wolk, 1988). Fragments near the site of the SA6 insertion were subcloned into pBluescript SK (Stratagene) and sequenced.

Reconstruction of the SA6 mutant.

A *Dra*I plasmid rescue of SA6 was linearized and ligated to an *Fsp*I fragment from pRL1130a (unpublished plasmids from the lab of C.P. Wolk), containing a streptomycin resistance marker and the *sacB* gene. The resulting plasmid was transferred to the AB5dr1 mutant and single recombinants were selected on medium containing neomycin (800 μg/mL). Several single recombinants were grown in liquid culture, then plated on media containing 5% sucrose and 100 μg/mL neomycin to select for double recombinants. Resulting double recombinants were Suc^r, Nm^r, Em^r, Cm^r, and Sp^s

Protein analysis.

Cultures of wild type 7120, the AB5 mutant and the SA6 mutant were treated with a 150 mM mixture of NaCl and KCl (equimolar) for 8 hrs. Cells were harvested by centrifugation, frozen in N_2 (*l*), resuspended in 1x Laemmli loading buffer and heated to 100° C for 5 min. Proteins were resolved on a SDS-PAGE gel (7.5-15% gradient) and silver stained.

Northern analysis.

Cultures (50 mL) were harvested by centrifugation and resuspended in 600 µL of 50 mM Tris, 10 mM EDTA, and 1% SDS. An equal volume of buffer-saturated phenol was added and the samples were mixed on a vortex mixer with sterile sand for 5 min. The phases were separated by centrifugation. The aqueous phase was transferred to a new tube and extracted twice with chloroform. Nucleic acids were precipitated on ice with Na-acetate pH 5.0 (0.2 M final concentration) and an equal volume of 2-propanol. The precipitate was sedimented by centrifugation in a micro-fuge at 14,000 rpm for 10 min. To remove DNA, the samples were resuspended in 4 M $LiCl_2$ and mixed on a vortex mixer for 5 min. RNA was precipitated by centrifugation at 14,000 rpm in a micro-centrifuge for 10 min., while the DNA remains in solution. The $LiCl_2$ step was repeated, then the RNA was resuspended in 500 µL of water, extracted once with phenol, and once with chloroform. The RNA was precipitated with Na-acetate and 2-propanol as before. RNA was subjected to electrophoresis in formaldehyde gels, transferred to nylon membranes and hybridized (Sambrook et al., 1989) with a *luxAB* probe.

Growth Curves.

Synchronously growing cultures of AB5 and SA6 were used to inoculate AA/8 N medium containing varying concentrations of an equimolar mixture of KCl and NaCl. After 7 days, cells from 1 mL of the culture were harvested by centrifugation. The chlorophyll a concentration was determined by measuring the absorption at 660 nm in one mL of 90% methanol (v/v).

RESULTS

Selection of mutants with insertions in salt-induced genes.

Insertions into salt inducible genes were identified by promoter trapping with a Tn5 derivative containing a promoter *luxAB* gene adjacent to the left border (Wolk et al., 1991). A photonic image of luciferase activity was obtained for filter-grown colonies. The filters were then transferred to medium containing an additional 100 mM NaCl. Colonies displaying elevated luciferase activity were identified by comparing photonic images taken prior to and subsequent to the hyper-osmotic shift (Figure 2.2). The luciferase activity in the AB5 mutant at different NaCl concentrations is shown in Figure 2.3. The genomic sequence adjacent to the AB5 insertion was nearly identical with the *lti2* gene (Figure 2.4), which was previously identified as a cold-inducible transcript in a related cyanobacterium, *Anabaena variablis* (Sato, 1993). *lti2* encodes a protein with similarity to α-glucanotransferases, but its function is unknown.

Second-site mutagenesis to identify regulatory components.

Second-site mutagenesis of the AB5dr1 strain was performed with the Tn5 derivative, pRL1058. Photonic images of salt-induced colonies were superimposed with

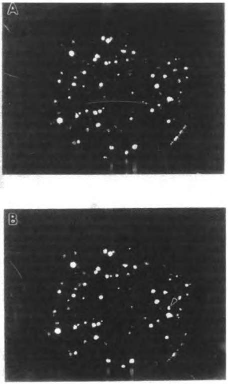


Figure 2.2- Photonic images of Tn5-1063 mutagenized colonies prior to and following a hyper-osmotic shift. The arrow indicates a mutant with an insertion in a salt-inducible gene

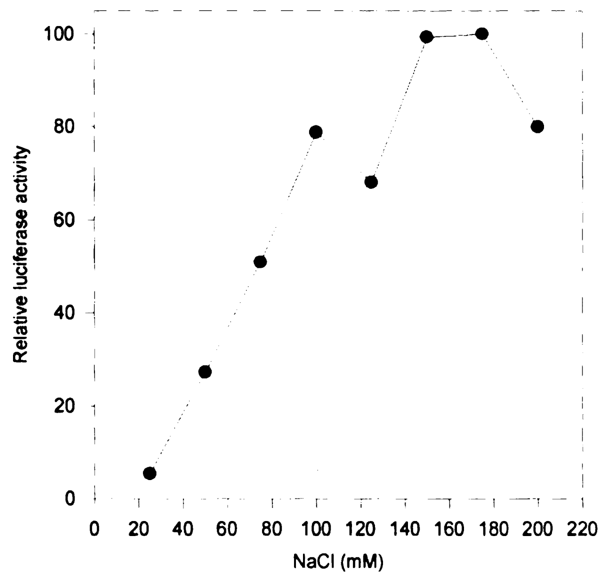


Figure 2.3- Luciferase activity in the AB5 mutant as a function of the NaCl concentration. Activity is expressed as a percentage of the maximum activity observed in this experiment.

AB5 right border

aacaagaccaatctaaaattgattggacactcttaggtaatgatcttaatcgtagtttttgattaccataaaggtttgattggtttacgt
aagaataataa

The sequence shown was identical with the nucleotide sequence of the *lti2* gene from *Anabaena variabilis.*

SD1 left border

atgttaattttagtcgttggaggttggcgggtaatggatgggaatctcagcattgggatgttggtggcatttcaaagcttgatgctg
agttttcagcagcctgtcaacgctttagtagatttcggtactactctccaggaattagaaggggatttaaatcgcttagatgatgat
gtgtt

The highest match from a blastx search of this sequence was: sp|q03727|coma_strpn
transport atp-binding protein coma
Poisson probability = 4.9e-06
Identities = 19/55 (34%), Positives = 34/55 (61%), Frame = +1

SA2 left border

gttaataataagggattacctgccagttcagcgatcgcatgggatgtatcaattccctatgcaggcgctctttttccgccttctatct
ccctcatcacaaaaagttaattcatgccagcgataaaatcttgaatttgttctggttctaaatcttg

No significant matches were found in the sequence databases.

SB11

cagga cgctacttgt ntataagagt caggtctcag ggtctacgat tttaacttctgtnccgggaa ttggttgtcc
ggntgtcccg ataaanttnc gccagggacg gcgcacgtnggtgactgggg atgtttcggt taaaccataa ccttgcaaaa
nctgcacgcc aataatttcaaaaaatgtnt cgatgtgttt tggtaatgca ccgccaccgc taattacttg
tttaaatcttcctcctgttg cttccttgac tttnccatac actaactttn gtcctaaaac atggaagggtaaaaangcaa
attctagtac cttagcgatt aancgttcca acgatgaagc atggagatgatctaaacttg taccttgagc aattntttgg
gctttantaa tattttcnac ntcattgncccantaaaaac cttaatcagg ccgttgtttn gtttccnggg ttgttcgggg
gacttngttttcancntcct tcataaatcg gttcccacat gcgggggtca

The highest match from a blastx search of this sequence was: sp|p39002|lcf3_Yeast
long-chain-fatty-acid--coa ligase 3 (ec 6.2.1.3) (Long-chain acyl-coa synthetase 3) (fatty acid activator 3).
Poisson probability = 1.5e-08
Identities = 28/79 (35%), Positives = 41/79 (51%), Frame = -2

Figure 2.4- Miscellaneous sequence data. The sequences were obtained from rescued plasmids using primers from the left and right ends of the Transposon

analog images to identify mutants with reduced luciferase expression (Figure 2.5). False positive resulting from a Tn5 insertion near the *luxAB* reporter gene were identified by Southern analysis (Figure 2.6). Several mutants had a reduced basal level of luciferase activity, but the relative induction of activity during salt-stress was similar to AB5. These mutants may be due spontaneous mutations in the *luxAB*. The SB12 mutant, which is impaired in phycobilisome synthesis (Cai et al., 1997), was also identified in this screen as a result of reduced viability.

One mutant, designated SA6, contained a normal basal level of activity, but failed to show any induction during salt stress (Figure 2.7). The mutation was reconstructed with an interposon based vector and shown to have the same phenotype. Analysis of proteins in this mutant revealed that several salt-induced proteins were no longer expressed in stressed cultures (Figure 2.8). Preliminary experiments indicate that the SA6 mutant is more sensitive to high salt concentrations than AB5 (Figure 2.9).

The *lti2* gene is induced by cold in *Anabaena variabilis* (Sato, 1993). The enhanced stability of luciferase at lower temperatures makes this a poor reporter gene for monitoring temperature induced changes in gene expression. Therefore, the expression of this gene in response to cold was investigated by northern analysis in the AB5dr1 and SA6 strains. There was a small increase in the abundance of the luciferase reporter trasncript in cold-stressed cultures, but there was no variation between the salt regulatory mutant, SA6, and the original reporter strain, AB5dr1 (Figure 2.10).

A genomic clone corresponding to the site of the SA6 insertion was identified and sequenced (Figure 2.11). An open reading frame (ORF) encoding a protein with sequence

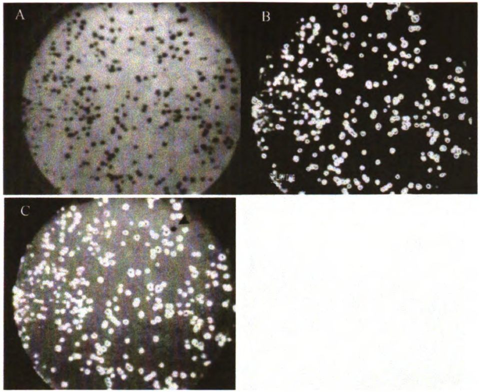


Figure 2.5- An photographic image (A) and a photonic image (B) of AB5dr1 colonies mutagenized with pRL1058. Filter-grown colonies were transferred to high salt media 5 hrs prior to obtaining the photonic image. The photographic image and the photonic image were superimposed (C) to identify mutants no longer displaying salt-induced luciferase activity.

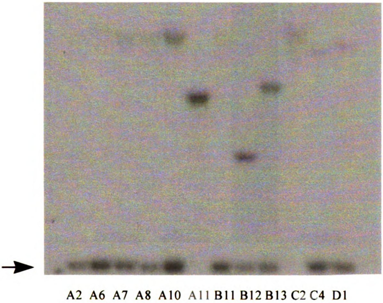


Figure 2.6- Southern analysis of genomic DNA from second-site mutants with pRL1063a as the probe. The arrows indicates an *EcoR*I fragment containing the *luxAB* reporter gene.

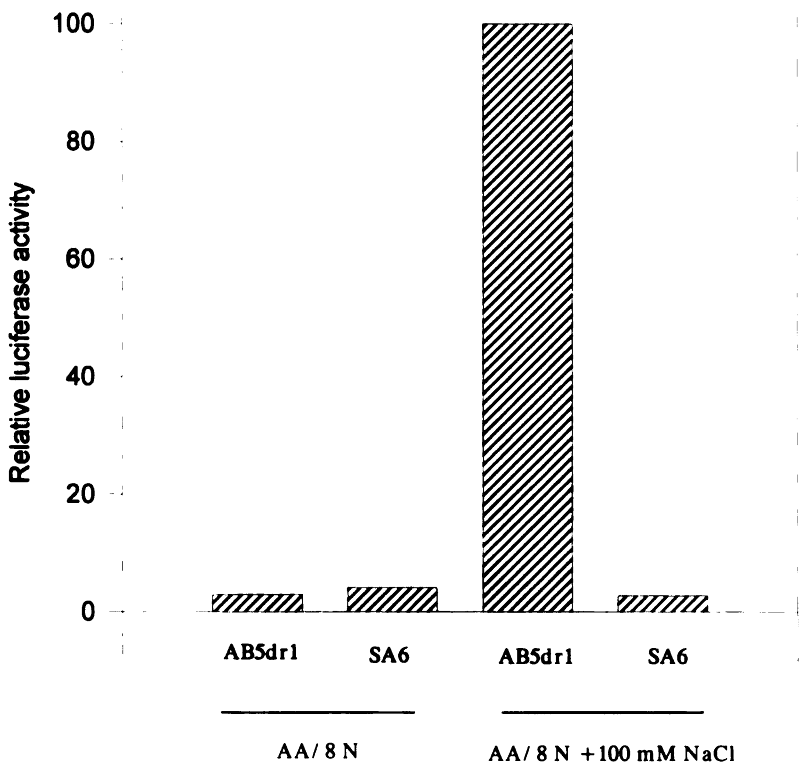


Figure 2.7- Luciferase activity in AB5dr1 and SA6 at different salt concentrations. The activity is expressed as a percentage of the maximum activity observed in this experiment.

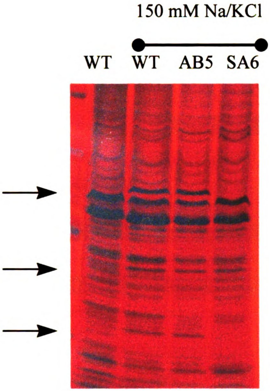


Figure 2.8- A silver stained SDS-PAGE gel. Samples are the wild type 7120, stressed wild type 7120, stressed AB5dr1, and stressed SA6. Stressed cultures were incubated with 150 mM NaCl and KCl (equimolar) for 8 hr.

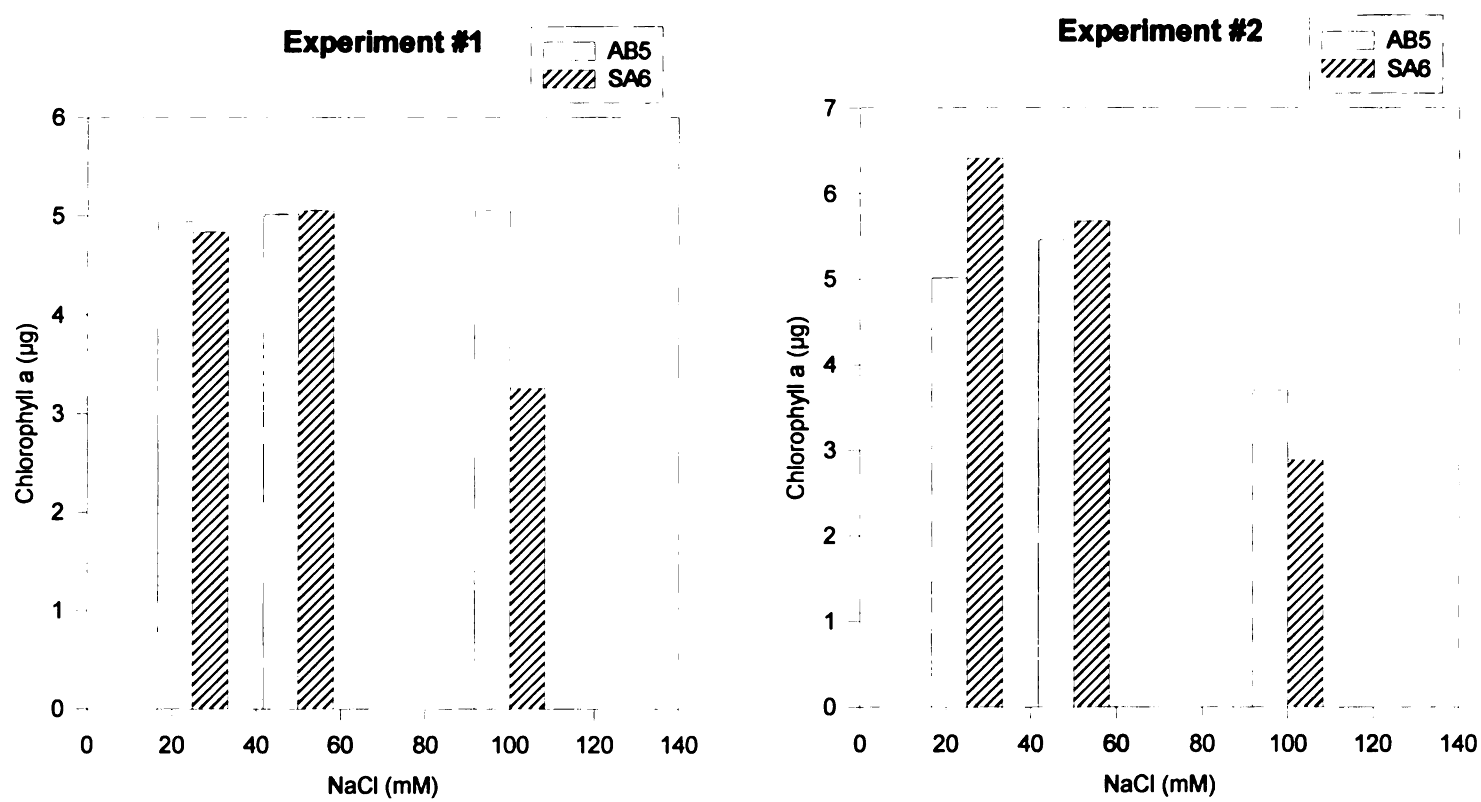


Figure 2.9- Growth of the AB5dr1 and SA6 mutants at different salt concentrations. The concentration of chlorophyll a was determined by measuring the absorption of methanol extracts at 660 nm.

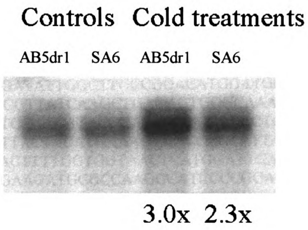


Figure 2.10- Northern analysis of *luxAB* expression in the SA6 and AB5dr1 strains

AAAACATTACTACCCAAAAATGATTTTACCTTTGCCAAAACCAATATATTGTC
ACAGTATTCTATATTTTCTTGTAAAATAGCGATATATCTATAGCCAGATTTTTA
TCTCTATCTAGACCCAAAAAAAAATAGATTTATTATTTCTATCGGTGGATGCA
GGCGCATCAAGATATCATCTATCTTTGGTTTGGGAAAATAATAGAAGTGACTG
AAAAAATAGCTTAGTTTGCAAGAAGCAATTAGACAAAGAGTGAGTGTAACG
ATGAGTGAAATCAGCATTATTTTAATTGAAGATCATGACCTAACCAGAATGGG
GCTAAGAGCTGCGTTACAGGCCAACACTGGCATCAAAGTAATTGGTGAAGCG
GCTAACGCCACTCAAGGGCTGAAACTTTTGGAAACGGCGAAGCCGGATGTAG
CGGTGGTAGATATTGGCTTGCCGGACATGGATGGTATTGAACTCACTCGCAA
▼GTTTAGGCGTTATCAAGCTGAGAGTGGGCAAACCCACACCAAGATTCTCAT
CCTGACAATGGATCATACCGAAGATGCGGTACTGGCGGCTTTTGCGGCTGGG
GCAGATTCTTACTACATGAAAGAAACCAGCATTAGTAGGCTAACAGAAGCAA
TTCAAGCTACTTTTGGTGGTAACTCATGGATTGATCCAGCGATCGCTAATGTA
GTATTACAGAAGATGCGCCAAGGCATCCCCGGAGAGAGCCAATCATCTGATA
AGCCCAAAACCGTCAAAATTGAGGCTCTGCCTTCTGAATACGAACAAGTATTA
GAAACCTACCCCCTTACACAACGGGAATTAGAAATTCTAGAGTTGATTGTTGC
TGGCTGTAGCAACGGTCAAATTGCGGAGAAACTTTATATTACTGTTGGTACGT
GCTTTGCGTTCTGGGTTAGTAGCTTAAACATGAAACCA**TAA**CGCACCTACCAA
AATTTAACACCCATACCCTAACCTTGGTTTCTCCGGCAATCAAGGTTTTAGCTT
TTTGCGCTAATTTCCTTAACGGTTATTACGGAATGGTTGCGAGTTGCTGAATA
ATTACCTTATAGCCCATCCACAGGTGTTAGAAGAGCTGCCATCATGCCAAACT
CCTGAGCGATAGGGTCTGCCAAGATAAGTAGTTGGTTCTGATGGTGAGAAATT

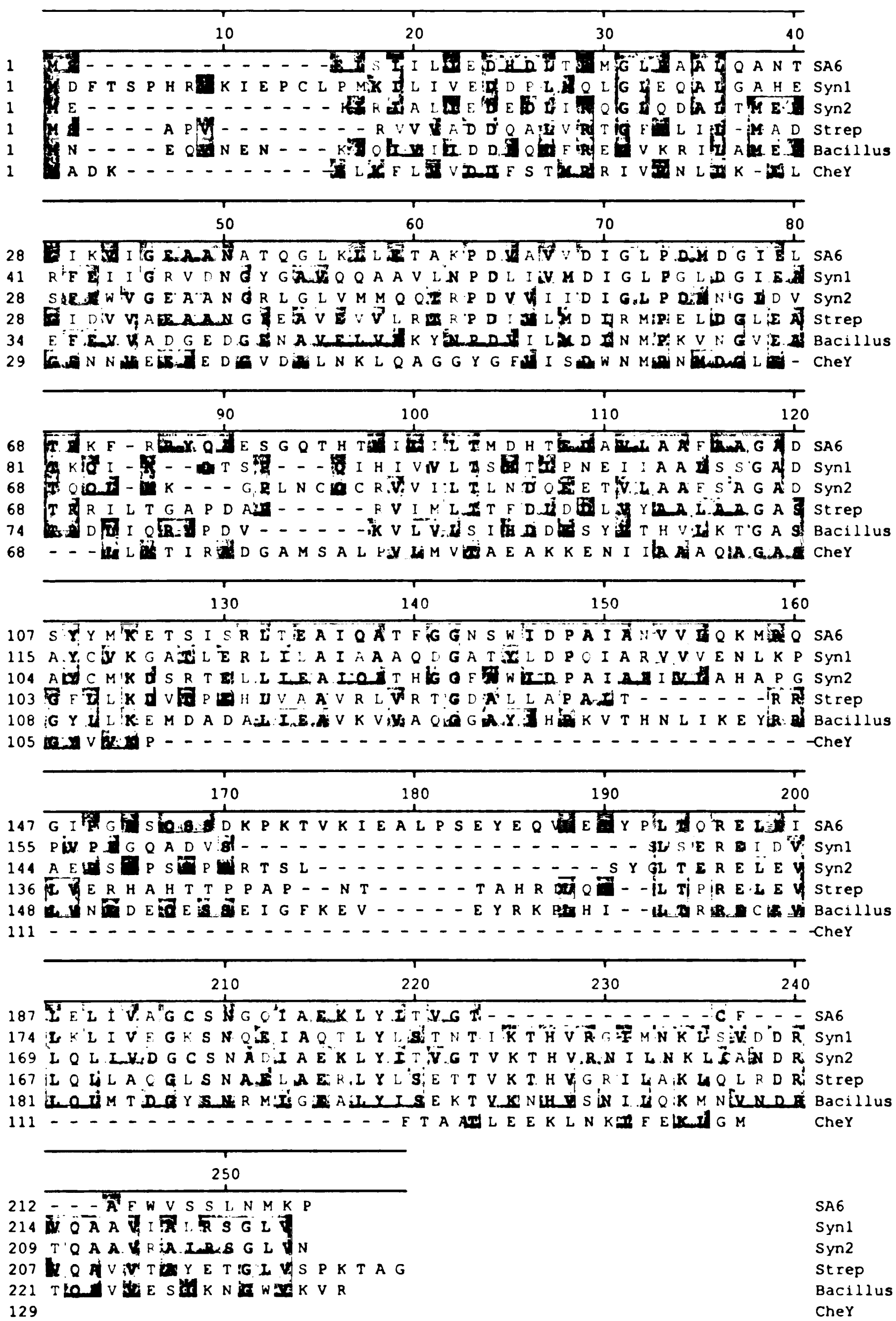
Figure 2.9- Nucleotide sequence at the site of the SA6 insertion. The start codon for the proposed start codon and stop codon are in **bold** and the site of the Tn5-1063 insertion is marked by ▼.

similarity to response regulators from two-component regulatory systems. The closest matches from the sequence databases were two hypothetical ORFs from the *Synechocystis* genome and a response regulator which controls the expression of extracellular proteases in *Bacillus brevis* (Louw et al., 1994) (Figure 2.12).

DISCUSSION

Analysis of proteins in stressed cultures of SA6 demonstrates that the expression of multiple genes is affected by the SA6 insertion. There were, however, several salt-induced proteins whose expression was unaffected in the SA6 mutant. This data indicates that multiple signal transduction pathways regulate the expression of salt-induced genes in *Anabaena* PCC 7120.

In the cyanobacterium *Anabaena variabilis*, the *lti2* transcript increases 40-fold within an hour of a 16° C temperature downshift (Sato, 1992). The induction of the *lti2* gene by both cold and salt stresses raises some interesting questions about the regulation and function of this gene. The expression of *luxAB* was analyzed by northern analysis in the AB5dr1 and SA6 mutants after a 14° C downshift. The increase in transcript levels was only 3-fold with the temperature downshift. The induction of the *lux AB* gene by cold is poor relative to the induction of *lti2* in *Anabaena variabilis*. This may indicate that this gene is not induced by cold in *Anabaena* PCC 7120. Alternatively, if the accumulation of *lti2* is not transcriptionally regulated, the northern analysis of *luxAB* expression would not properly reflect changes in the expression of this gene. In the cyanobacterium *Synechococcus*, the induction of the *desA* and *desB* genes in response to cold is, in part, due to changes in mRNA stability (Sakamoto and Bryant, 1997). This may also explain



Decoration 'Decoration #1': Shade (with black at 40% fill) residues that match the Consensus exactly.

Figure 2.12- The deduced amino acid sequence of SA6 aligned with two hypothetical ORFs from the *Synechocystis* genome (gi-1653479 and gi-1652813), an ORF from *Streptomyces lividans* (gi-1498492), and the *degU* gene product from *Bacillus brevis* (sp-p54662). The *CheY* gene product from *E. coli* (gi-1736535) is also included, because the crystal structure is known and is often used as a reference in defining critical (Volz, 1993).

the poor induction of the *luxAB* transcript by cold in the AB5dr1 strain relative to the *lti2* transcript in *Anabaena variablis*.

The deduced sequence for the amino terminus of SA6 is similar to the sequence of many response regulators. This domain interacts with the sensory kinase and is highly conserved in other response regulators (Gross et al., 1989). The closest match from the sequence databases is a *Synechocystis* ORF with unknown function (Kaneko et al., 1996). It is uncertain wether this *Synechocystis* gene is a functional homolog of SA6. A potential response regulator for salt stress responses in *Synechocystis* has previously been identified (Hagemann et al., 1996), but shares only moderate sequence similarity with SA6. The secondary structure predictions for the carboxy terminus of SA6 suggests a helix-loop-helix DNA binding motif, which is found in other response regulators and is consistent with a role in gene activation.

Other elements in the salt-induced signal transduction pathway have yet to be identified. Since both ionic and and non-ionic osmotica induce the expression of AB5, the sensory kinase regulating the expression of this gene may perceive changes in turgor. The identification and characterization of the sensory kinase with the second-site mutagenesis strategy would be useful for further studies.

REFERENCES

Allen MB, Arnon DI (1955) Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. **30:** 366-372

Anandan S, Nalty MS, Cogdell DE, Golden SS (1996) Identification of 2 classes of transcriptional regulator genes in the cyanobacterium *Synechococcus* sp strain PCC 7942. Arch Microbiol **166:** 58-63

Apte SK, Bhagwat AA (1989) Salinity-stress-induced proteins in two nitrogen-fixing *Anabaena* strains differentially tolerant to salt. J Bacteriol **171:** 909-915

Apte S.K., Haselkorn R (1990) Cloning of salinity stress-induced genes from the salt-tolerant nitrogen-fixing cyanobacterium *Anabaena torulosa*. Plant Mol Biol **15:** 723-733

Black TA, Wolk CP (1994) Analysis of a Het$^-$ mutation in *Anabaena* sp. strain PCC 7120 implicates a secondary metabolite in the regulation of heterocyst spacing. J Bacteriol **176:** 2282-2292

Campbell EL, Hagen KD, Cohen MF, Summers ML, Meeks JC (1996) The devR gene product is characteristic of receivers of two-component regulatory systems and is essential for heterocyst development in the filamentous cyanobacterium *Nostoc* sp. strain ATCC 29133. J Bacteriol **178:** 2037-2043

Chiang GG, Schaefer MR, Grossman AR (1992) Complementation of a red-light-indifferent cyanobacterial mutant. Proc Natl Acad Sci USA **89:** 9415-9419

Close TJ, Lammers PJ (1993) An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins. Plant Physiol **101:** 773-779

Curry J, Walker-Simmons MK (1993) Sequence analysis of wheat cDNAs for abscisic acid-responsive genes expressed in dehydrated wheat seedlings and the cyanobacterium, *Anabaena. In* Plant Responses to Cellular Dehydration During Environmental Stress. Ed. Close T.J., Bray E.A. American Society of Plant Physiologists, Rockville, MD

Elhai J, Wolk CP (1988) Conjugal transfer of DNA to cyanobacteria. Methods Enzymol **167:**747-754

Fernandes TA, Iyer V, Apte SK(1993) Differential responses of nitrogen-fixing cyanobacteria to salinity and osmotic stresses. Appl Env Micro **59:** 899-904

Gross R, Aricò B, Rappuoli R (1989) Families of bacterial signal transducing proteins. Mol Microbiol **3:**1661-1667

Hagemann M, Richter S, Zuther E, Schoor A (1996) Characterization of a glucosylglycerol-phosphate-accumulating, salt-sensitive mutant of the cyanobacterium *Synechocystis* sp. strain PCC-6803. Arch Microbiol **166:** 83-91.

Hagemann M, Golldack D, Biggins J, Erdmann N (1993) Salt-dependent protein phosphorylation in the cyanobacterium *Synechocystis* PCC 6803. FEMS Microbiol Lett **113:** 205-210

Hagemann M, Zuther E (1992) Selection and characterization of mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 unable to tolerate high salt concentrations. Arch Microbiol **158:** 429-434

Haro R, Garciadeblas B, Rodriguez-Navarro A (1991) A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett **291:** 189-191

Joset F, Jeanjean R, Hagemann M (1996) Dynamics of the response of cyanobacteria to salt stress: deciphering the molecular events. Physiol Plantarum **96:** 738-744

Kamamaru K, Kashiwagi S, Mizumo T (1993) The cyanobacterium *Synechococcus* sp. PCC 7942 possesses genes encoding cation-transporting P-type ATPases. FEBS Lett **330:** 99-104

Mikkat S, Hagemann M, Shoor A (1996) Active transport of glucosylglycerol is involved in salt adaptation of the cyanobacterium *Synechocystis* sp. PCC 6803. Microbiology **142:** 1725-1732

Louw ME, Reid SJ, James DM, Watson TG (1994) Cloning and sequencing the *DegS-DegU* operon from an alkalophilic *Bacillus brevis*. Appl Microbiol Biotechnol **42:** 78-84

Maeda T, Wurgler-Murphy SM, Saito (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369:** 242-245

Nagaya M, Aiba H, Mizuno T (1994) The *sphR* product, a two-component system response regulator protein, regulates phosphate assimilation in *Synechococcus* sp. strain PCC 7942 by binding to two sites upstream from the *phoA* promoter. J Bacteriol **176:** 2210-2215

Pabo CO, Sauer RT (1992) Transcription factors: structural families and principles of DNA recognition. Annu Rev Biochem **61:** 1053-1095

Packer L, Spath S, Martin JB, Rory C, Bligny P (1987) ^{23}Na and ^{31}P NMR studies of the effects of salt stress on the freshwater cyanobacterium *Synechococcus* 6311. Arch Biochem Biophys **256:** 354-361

Potts M (1994) Desiccation tolerance of prokaryotes. Microbiol Rev **58:** 755-805

Reed RH, Richardson DL, Warr SRC, Stewart WDP (1984) Carbohydrate accumulation and osmotic stress in cyanobacteria. J Gen Microbiol 130: 1-4

Reed RH, Stewart WDP (1988) The response of cyanobacteria to salt stress. Methods

Enzymol **167:** 217-231

Sakamoto T, Bryant DA (1997) Temperature-regulated mRNA acumulation and stabilization for fatty acid desaturase genes in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Mol Microbiol **23:** 1281-1292

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sato N (1992) Cloning of a low-temperature induced gene *lti2* from the cyanobacterium *Anabaena variabilis* M3 that is homologous to alpha-amylases. Plant Mol Biol **18:** 165-170

Swanson RV, Alex LA, Simon MI (1994) Histidine and aspartate phosphorylation: two-component systems and the limits of homology. Trends Biochem Sci **19:** 485-490

Volz K (1993) Structural conservation in the CheY superfamily. Biochemistry **32:** 11741-11753

Wolk CP, Cai Y, Panoff J-M (1991) Use of a transposon with luciferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc Natl Acad Sci USA **88:** 5355-5359

Wolk CP, Vonshak A, Kehoe P, Elhai J (1984) Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. Proc Natl Acad Sci USA **81:** 1561-1565

Chapter 3

Biochemical characterization of the *aba2* and *aba3* mutants of Arabidopsis.

ABSTRACT

Abscisic acid (ABA) deficient mutants, in a variety of species, have been identified by screening for precocious germination and a wilty phenotype. Two new mutants, *aba2* and *aba3*, have recently been identified (Léon-Kloosterziel et al., [1996], Plant J 10: 655-661). The biochemical characterization of these mutants is presented here. Protein extracts from *aba2* and *aba3* plants displayed a greatly reduced ability to convert xanthoxin to ABA relative to the wild type. The next putative intermediate in ABA synthesis, ABA-aldehyde, was efficiently converted to ABA by extracts from *aba2*, but not by extracts from *aba3* plants. This indicates that the *aba2* mutant is blocked in the conversion of xanthoxin to ABA-aldehyde and *aba3* is impaired in the conversion of ABA-aldehyde to ABA. Extracts from the *aba3* mutant also lacked several additional activities which require a molybdenum cofactor (Moco). Nitrate reductase utilizes a Moco but its activity was unaffected in extracts from *aba3* plants. Moco hydroxylases in animals require a desulfo moiety of the cofactor. Under anaerobic conditions, a sulfur ligand can be added to the Moco by treatment with Na_2S and dithionite. Treatment of *aba3* extracts with Na_2S restored ABA-aldehyde oxidase activity. Therefore, the genetic lesion in *aba3* appears to be in the introduction of sulfur into the Moco.

INTRODUCTION

ABA is a sesquiterpenoid plant growth regulator involved in the induction of seed dormancy and adaptation to a variety of stresses (Zeevaart and Creelman, 1988). The regulation of these physiological processes is in part due to *de novo* synthesis of ABA. Thus, an understanding of the ABA biosynthetic pathway and its regulation is essential for an appreciation of the factors mediating plant stress responses. The characterization of ABA-deficient mutants has been helpful in elucidating the biosynthetic pathway. For example, the *aba1* mutant, which is impaired in the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Duckham et al., 1991; Rock and Zeevaart, 1991), provided definitive evidence that ABA is derived from an epoxy-carotenoid precursor in higher plants.

The first committed step in ABA biosynthesis appears to be the oxidative cleavage of an epoxy-carotenoid precursor to form xanthoxin (Parry et al., 1988). Following the cleavage reaction, xanthoxin is rapidly converted to ABA by a series of ring modifications and the oxidation of the aldehyde to a carboxylic acid. Sindhu and Walton (1987) proposed a pathway based upon feeding potential intermediates to a cell-free system and monitoring their conversion to ABA. If a substrate was not efficiently converted to ABA in the cell-free system it was considered an unlikely intermediate *in vivo*. The results from these experiments suggest that the ring transformations occur first to produce ABA-aldehyde and the oxidation of the aldehyde is the final step (Figure 3.1). During the conversion of xanthoxin to ABA-aldehyde three modifications of the ring occur: oxidation of the 4'-hydroxyl to a ketone, desaturation of 2'-3' bond, and opening of the

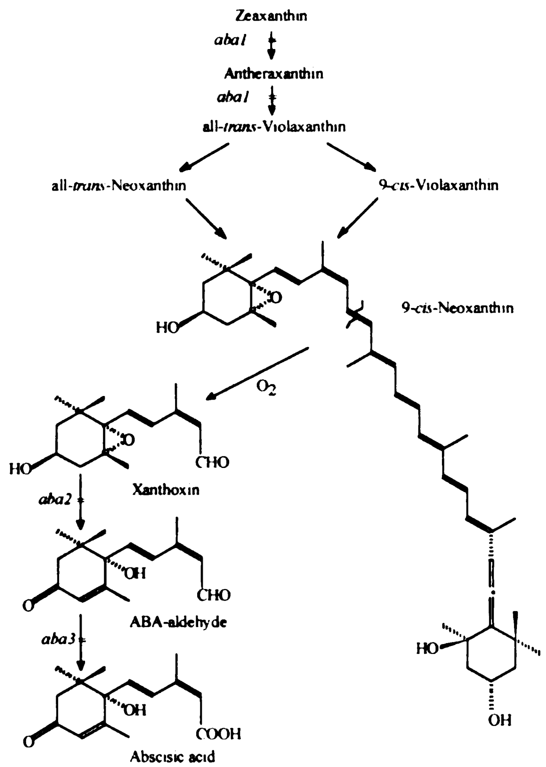


Figure 3.1- Proposed pathway of ABA biosynthesis. The biochemical lesions in the *aba1*, *aba2,* and *aba3* mutants of *Arabidopsis thaliana* are indicated.

epoxide ring.

Mutants that are impaired in the ring transformation have not yet been reported. A number of mutants have been identified in the final step of ABA biosynthesis, the oxidation of the aldehyde to the carboxylic acid (Taylor, 1991): *flc* and *sit* in tomato, *dr* in potato, *aba1* in *Nicotiana plumbaginifolia*, and *nar2a* in barley. The *nar2a* mutant in barley was shown to lack ABA-aldehyde oxidase, xanthine dehydrogenase, and nitrate reductase activities (Walker-Simmons et al., 1989). This pleiotropic phenotype is the result of a lesion in the synthesis of the Moco, which all three enzyme activities require. Surprisingly, precocious germination and a wilty phenotype have not been reported for Moco mutants, *cnx*, in *Arabidopsis thaliana*. Presumably, the lesions in the Arabidopsis mutants are sufficiently leaky to allow for normal ABA biosynthesis.

In tobacco, an ABA-deficient mutant lacking aldehyde oxidase and xanthine dehydrogenase, but not nitrate reductase, has been identified (Leydecker et al., 1995). The phenotype of the tobacco mutant may be explained by the existence of two forms of the Moco in eukaryotes (Wahl and Rajagopalan, 1982). Moco hydroxylases, such as xanthine dehydrogenase and aldehyde oxidase, use a desulfo form of the Moco, while reductive dehydroxylases, such as nitrate reductase, utilize a dioxo form of the Moco. The lesion in the barley *nar2a* mutant must occur at an early step in the pathway, so that it affects both forms of the Moco. The lesion in the tobacco mutant appears to be in the modification of the Moco to form the desulfo moiety. In *Drosophila*, a mutant impaired in the "sulfuration" of the Moco has been identified (Wahl et al., 1982).

The characterization of ABA-deficient mutants has been valuable in elucidating the function of ABA and the pathway of ABA biosynthesis. The biochemical characterization of two new mutants in *Arabidopsis thaliana*, *aba2* and *aba3* (Léon-Kloosterziel et al., 1996), is described below.

MATERIALS AND METHODS

Plant Material.

Columbia (WT) and the mutants *aba2-1* (isolation J14) and *aba3-1* (isolation J25) were grown in a 9-hour photoperiod as previously described (Rock and Zeevaart, 1991). Rosettes were frozen in liquid N_2 and stored at -80°C until extraction.

Preparation of enzyme extracts.

Leaves were ground with a mortar and pestle in 0.2 M KPi, pH 7.5, containing 10 mM DTT (3 mL/g leaf material). The extracts were filtered through four layers of cheesecloth and centrifuged at 12,000*g* for 10 min at 4°C. Proteins were precipitated from the supernatant with ammonium sulfate (80% saturation). The ammonium sulfate precipitate was resuspended in 50 mM KPi, pH 7.5, and desalted on a PD-10 size exclusion column according to the manufacturer's instructions (Pharmacia).

Activity gels.

Extracts were subjected to native gel electrophoresis and aldehyde oxidase activities were detected with the substrate heptaldehyde (Walker-Simmons et al., 1989). As previously reported, xanthine dehydrogenase activity is difficult to detect in crude extracts from *Arabidopsis thaliana* (LaBrie et al., 1992). However, the activity was easily detected on activity gels when a 16%-32% ammonium sulfate fraction was analyzed.

Preparation of substrates.

Violaxanthin and neoxanthin isolated from spinach leaves were oxidized with $ZnMnO_4$ (Taylor and Burden, 1972). Xanthoxin was then purified by normal phase HPLC on a µPorasil semipreparative column (0.78 x 30 cm) (Waters). Xanthoxin was eluted with a linear gradient of 10 to 100% (v/v) ethyl acetate in hexane in 72 min at a flow rate of 2.5 mL min^{-1}. A xanthoxin fraction was collected from 39-42 min and dried under a stream of N_2. Xanthoxin was further purified by reverse phase HPLC on a µBondapak C_{18} semipreparative column (Waters) with a linear gradient of 10 to 60% (v/v) ethanol in water over 40 min at 2.5 mL min^{-1}. A fraction containing *cis*-xanthoxin was collected from 23 to 25 min . The identity and purity of xanthoxin was confirmed by GC-MS of the TMSi derivative (Gaskin and MacMillan, 1992). ABA-aldehyde was provided by Hoffmann-LaRoche.

Conversion of xanthoxin and ABA-aldehyde to ABA.

Enzyme assays contained 50 mM KPi, 0.25 mM EDTA, 1 mM PMSF, 1 mM NADP, and the appropriate substrate in a total volume of 200 µL. Following a 1-hour incubation at 28°C, 400 µL of acetone was added to stop the reactions. Proteins were pelleted by centrifugation at 10,000*g* and [^{3}H-]ABA was added to the supernatant as an internal standard. The acetone was evaporated under vacuum, one mL of 2% acetic acid (v/v) was then added to each sample, and ABA was partitioned three times into ethyl acetate. The ethyl acetate fractions were pooled, dried under a stream of N_2, and ABA was methylated with diazomethane. Me-ABA was purified by normal phase HPLC (Creelman et al., 1987) and analyzed on a Hewlett Packard 6890 GC equipped with an electron capture detector. Samples were injected on a HP-5 capillary column (Hewlett-Packard) and chromatographed iso-thermally at 188°C. Quantitation of Me-ABA was performed as previously described (Cornish and Zeevaart, 1985).

Analysis of ABA-alcohol in the *aba3* mutant.

Plant tissue, 0.5 g dry weight, was extracted in 80% MeOH (aq). The methanol was evaporated under vacuum and the remaining water was partitioned three times with diethyl ether. The diethyl ether fractions contained ABA-alcohol, while the ABA-alcohol glucoside remained in the aqueous phase. The glucoside was hydrolyzed with almond glucosidase (Sigma) in 0.1 M KPi pH 4.7 and ABA-alcohol was partitioned into diethyl ether. The ABA-alcohol from both fractions was injected on a normal phase HPLC column equilibrated with 90:10 hexane and ethyl acetate. The column was eluted with a gradient to 80% ethyl acetate over 25 min at 2.5 mL min $^{-1}$. A fraction corresponding to ABA-alcohol was collected at 24-25 min. and dried under a stream of N_2. ABA-alcohol was analyzed by GC equipped with an electron capture detector.

Inactivation and reconstitution of ABA-aldehyde oxidase activity.

In extracts of WT Columbia, aldehyde oxidase activity was inactivated by treating extracts with 20 mM KCN for one hour at room temperature. Following the incubation, CN^- was removed on a spin column, which was prepared by filling a 1 mL syringe with G-25 Sephadex equilibrated in 50 mM KPi, pH 7.5. For reactivation, extracts were made anaerobic by degassing under vacuum and purging with anaerobic hydrogen several times. Anaerobic solutions of dithionite and Na_2S were added through a septum and the extracts were incubated at 37°C for 30 min. Following the incubation dithionite and Na_2S were removed on a G-25 Sephadex spin column and ABA-aldehyde oxidase activity was assayed as described above.

Analysis of polar metabolites in the *aba3* mutant.

Rosettes were frozen in liquid N_2 and lyophilized. The tissue was extracted with 80% MeOH containing BHT (0.1 g/ L). The MeOH was evaporated under vacuum and the pH of the remaining aqueous portion was adjusted to 3.5 with acetic acid. The extract was then partitioned three times with an equal volume of diethyl ether. The aqueous phase was concentrated under vacuum, filtered through a 0.45 µm HA filter (Millipore), and injected on a semipreparative (0.78 x 30 cm) µBondapak C_{18} HPLC column (Waters). The column was eluted with a linear gradient of 0 to 50% (v/v) ethanol in water over 35 min at a flow rate of 2.5 mL min^{-1}.

RESULTS

Conversion of xanthoxin and ABA-aldehyde to ABA by cell-free extracts.

Previous work (Léon-Kloosterziel et al., 1996) has shown that the *aba2* and *aba3* mutants are ABA-deficient. Analysis of total carotenoids in *aba2* and *aba3* indicated no variation when compared to the WT (Figure 3.2). This indicates that both mutants are impaired in the later steps of ABA biosynthesis. Cell-free extracts of *Arabidopsis thaliana* were prepared to monitor the conversion of xanthoxin to ABA. The requirements for activity were similiar to those reported for cell-free preparations from other species (Sindhu and Walton, 1987). NADP was the only cofactor necessary for activity. DTT, glutathione, or cysteine enhanced the conversion of xanthoxin to ABA but were omitted from the assays, because they caused the non-enzymatic *cis, trans* isomerization of

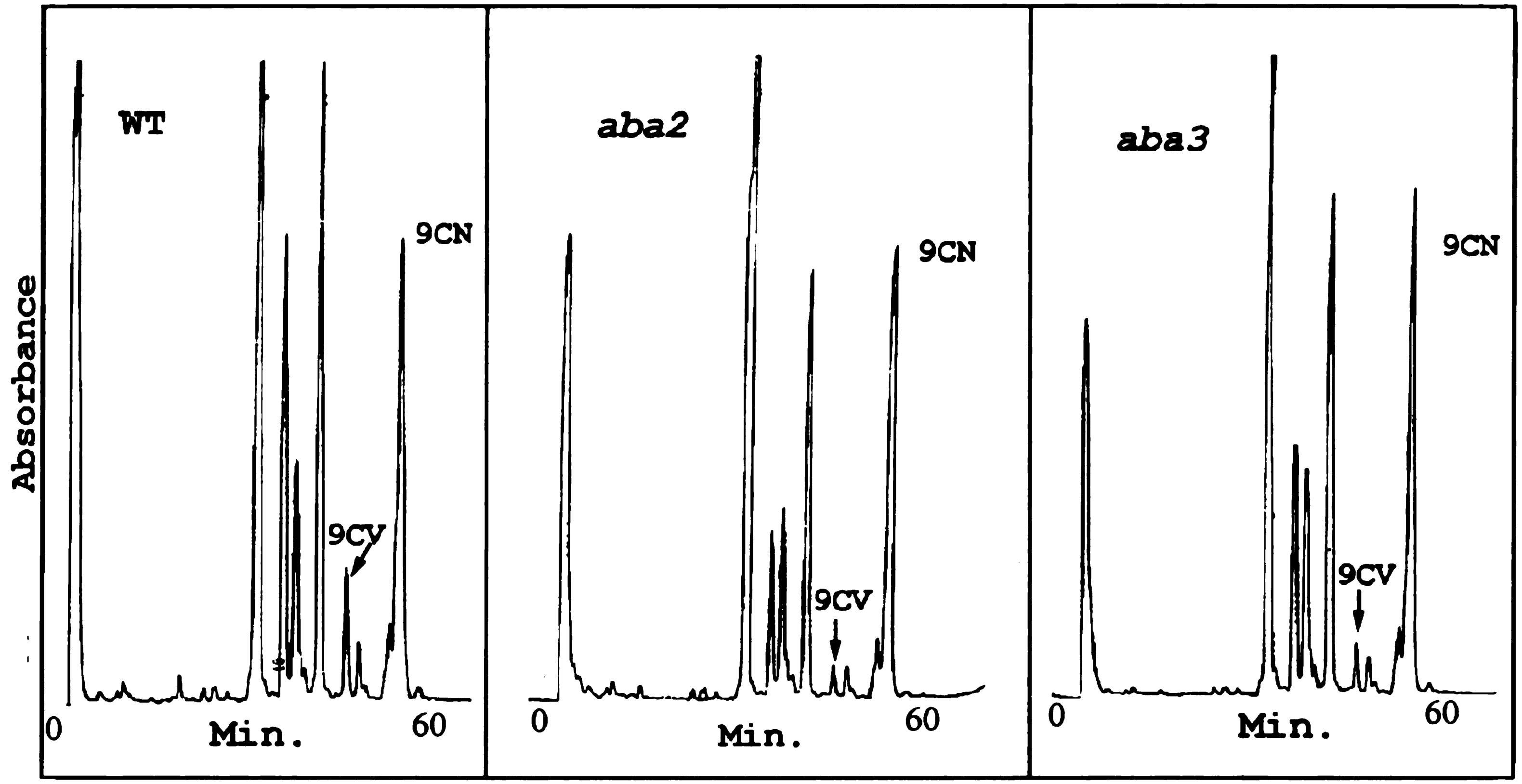


Figure 3.2- Carotenoid analysis of the WT Columbia, *aba2*, and *aba3* plants. 9-*cis*-violaxanthin (9cV) and 9-*cis*-neoxanthin are indicated the chromatograms.

xanthoxin (data not shown). No variation in activity was observed when extracts were made from turgid or dehydrated leaves (Table 3.1).

The conversion of xanthoxin to ABA by *aba2, aba3,* and WT extracts was measured as a function of protein concentration (Figure 3.3). Cell-free extracts from the two mutants, *aba2* and *aba3*, showed a substantially reduced ability to convert xanthoxin to ABA. ABA-aldehyde was also fed to cell-free extracts to monitor its conversion to ABA. Extracts of *aba2* converted ABA-aldehyde to ABA as efficiently as WT extracts (Figure 3.4). No ABA was detected in assays with extracts from *aba3* plants at the protein concentrations indicated in figure 3.4. Tobacco and tomato mutants, which are unable to oxidize ABA-aldehyde to ABA accumulate *trans* -ABA-alcohol and a glucoside of *trans*-ABA-alcohol (Linforth et al., 1987; Leydecker et al., 1995). The *flc* and *sit* mutants of tomato (Taylor et al., 1988) and the *dr* mutant in potato are capable of converting exogenous *cis*-ABA-aldehyde to these compounds (Duckham et al., 1989). The *aba3* mutant also accumulates *trans*-ABA-alcohol and the glucoside of *trans*-ABA alcohol (Figure 3.5). In wild type Columbia, *trans*-ABA-alcohol was also detectable, but at much lower concentrations than in *aba3* plants (data not shown).

The *aba3* mutant has a pleiotropic phenotype.

The lesion in *aba3* may result from a mutation in the apoprotein which converts ABA-aldehyde to ABA or in an enzyme involved in the synthesis of the Moco (Walker-Simmons et al., 1989; Leydecker et al., 1995). The activities of aldehyde oxidase, xanthine dehydrogenase, and nitrate reductase, which all require a Moco, were measured in extracts from WT Columbia and *aba3* plants. Aldehyde oxidase activity was tested on

Table 3.1- The conversion of xanthoxin (100 ng per assay) to ABA (ng) by cell-free extracts of turgid and stressed leaves.

	Protein	
	400 µg	800 µg
Turgid	6.04±1.2	7.69±1.0
Stressed	5.75±.2	7.85±1.2

Table 3.2- Nitrate reductase activity (nmol NO_2^- min $^{-1}$ mg protein^{-1} ± S.D.) in WT Columbia and *aba3* extracts.

Genotype	Nitrate reductase activity
Columbia	27.7±0.9
aba3	23.1±4.5

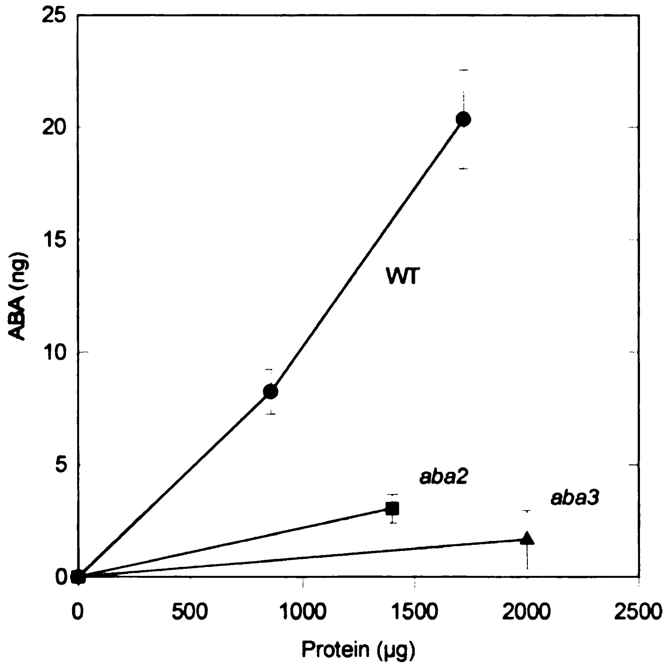


Figure 3.3- Conversion of xanthoxin to ABA by protein extracts from WT Columbia, *aba2*, and *aba3* plants. 100 ng of xanthoxin was added per assay.

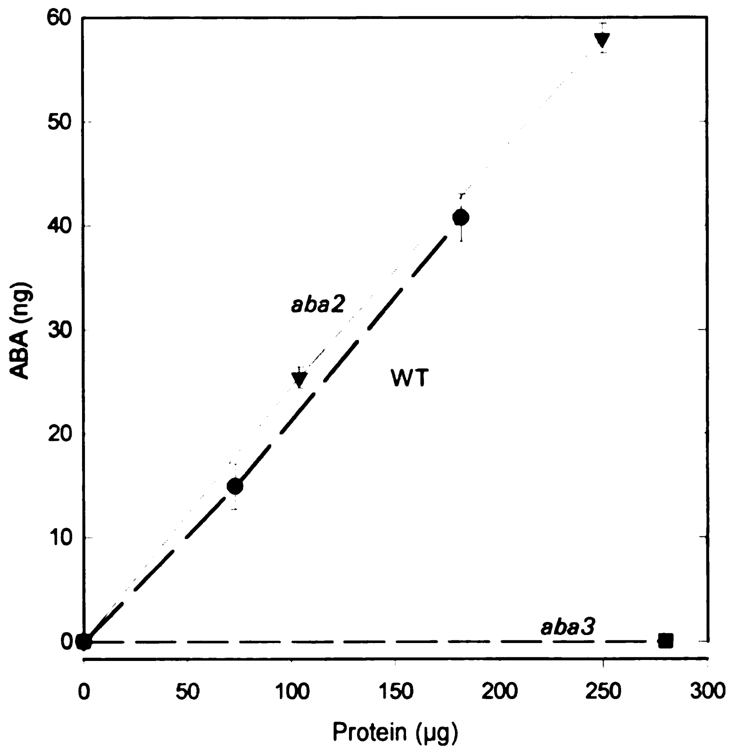


Figure 3.4- Conversion of ABA-aldehyde to ABA by protein extracts of WT Columbia, *aba2*, and *aba3* plants. 50 ng of ABA-aldehyde was added per assay.

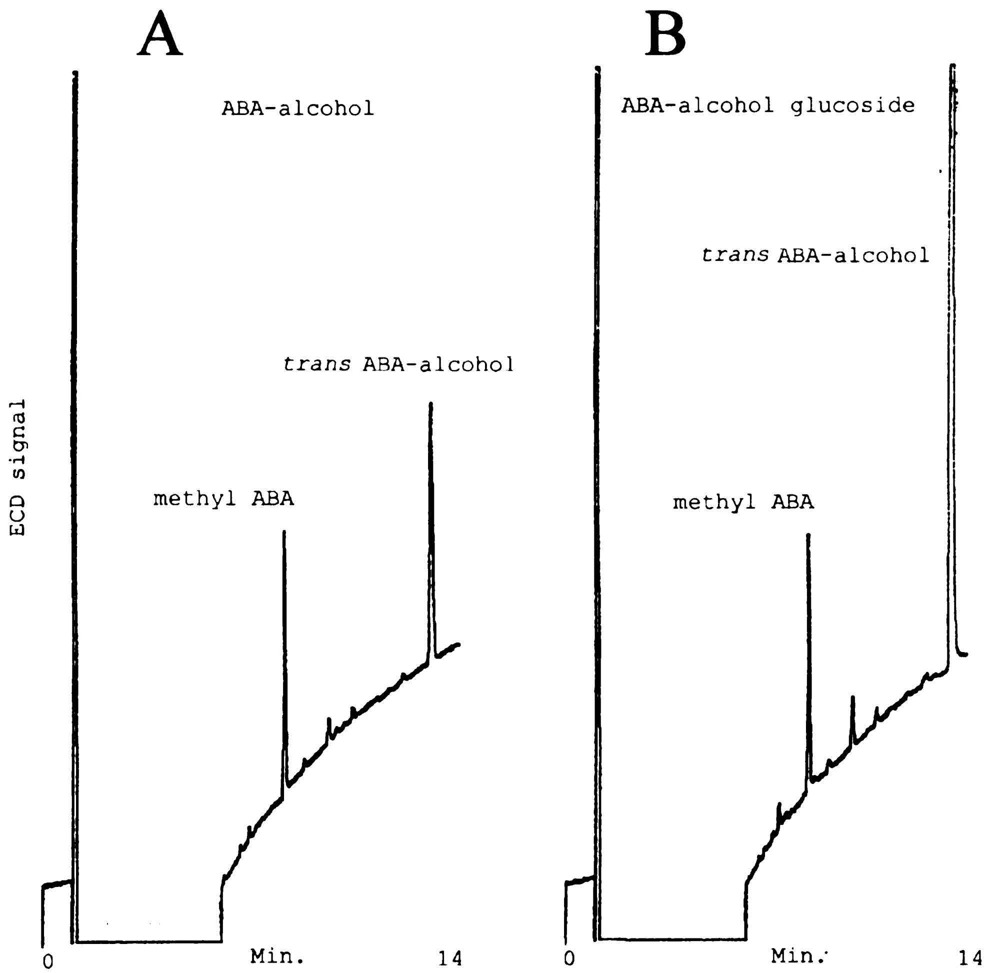


Figure 3.5- The accumulation of *trans*-ABA-alcohol in the *aba3* mutant. (A) *trans*-ABA-alcohol from the diethyl ether fraction was dissolved in 500 µL, and 1 µL was injected on the GC; 100 pg/µL methyl-ABA was added as a reference standard. (B) The *trans*-ABA-alcohol hydrolyzed with glucosidase, 1 µL of 1000 µL was injected with 100pg/µL methyl-ABA as a reference standard.

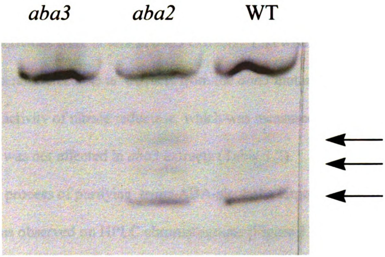


Figure 3.6- Aldehyde-oxidase activity gel with protein extracts of WT Columbia, *aba2*, and *aba3* (80 µg of protein was loaded per lane). The aldehyde oxidase activities are indicated with arrows. The most intense band (at the top of the gel) is a staining artifact, which also occurs in the absence of the heptaldehyde substrate.

an enzyme activity gel using heptaldehyde as substrate (Figure 3.6). Both Columbia and *aba2* extracts contained three activities capable of oxidizing heptaldehyde. All three aldehyde oxidase activities were absent in *aba3* extracts. Likewise, xanthine dehydrogenase was not detectable in extracts from the *aba3* mutant in an activity gel (Figure 3.7). The activity of nitrate reductase, which was measured according to Wray and Filner (1970), was not affected in *aba3* extracts (Table 3.2).

During the process of purifying *trans*-ABA-alcohol a large UV absorbing peak, unique to *aba3*, was observed on HPLC chromatograms (Figure 3.8). The accumulation of this compound, which has not been identified, is another example of the pleiotropic phenotype of the *aba3* mutant.

The pleiotropic phenotype of *aba3* results from a defect in Moco biosynthesis. In eukaryotes, a dioxo and a desulfo moiety of the Moco have been identified (Wahl and Rajagopalan, 1982). Nitrate reductase activity is unaffected in the mutant; suggesting that only one form of the Moco is absent. The two forms of the Moco can be chemically converted from one to the other (Wahl and Rajagopalan, 1982). Treatment with CN^- will hydrolyze the sulfur ligand. Under strictly anaerobic conditions a sulfur ligand may be added to the Moco by treatment with dithionite and Na_2S. Extracts of WT Columbia incubated with CN^- resulted in the inactivation of ABA-aldehyde oxidase activity, which could subsequently be restored by treatment with Na_2S (Figure 3.9). The activity of ABA-aldehyde oxidase in *aba3* could be activated by treating extracts with dithionite and Na_2S.

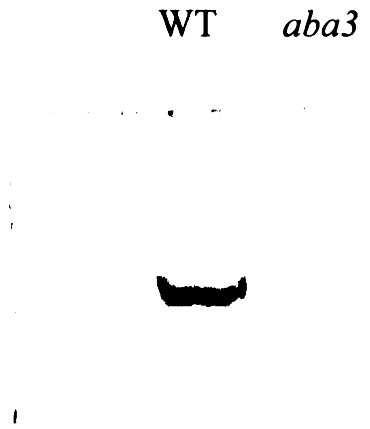


Figure 3.7- Xanthine dehydrogenase activity gel with protein extracts of WT Columbia and *aba3*: 50 µg of a 16-32% ammonium sulfate fraction were loaded per lane.

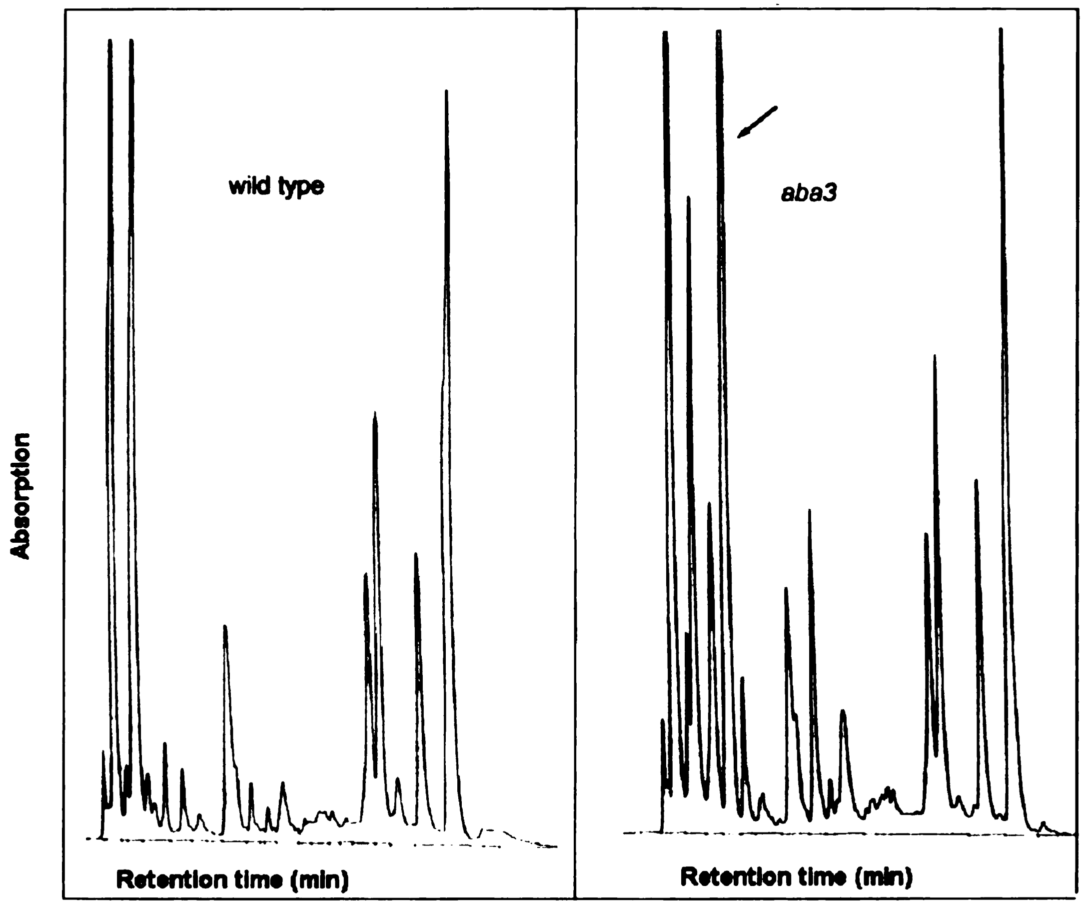


Figure 3.8- Reverse-phase HPLC chromatogram of aqueous extracts (see materials and methods) from WT Columbia and *aba3*. The arrow indicates a compound which only accumulates in the *aba3* mutant.

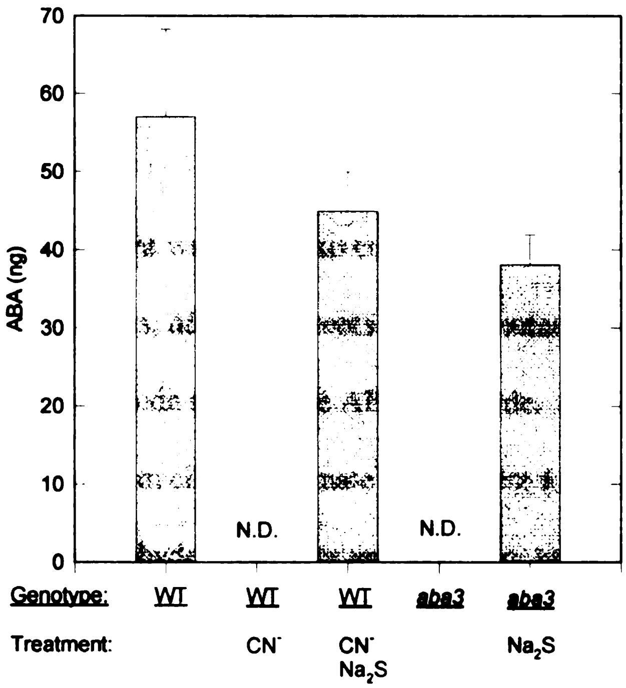


Figure 3.9- Inactivation of ABA-aldehyde oxidase activity in WT Columbia with KCN and reconstitution of activity in *aba3* by treatment with Na_2S and dithionite. N.D. (not detected). 50 ng of ABA-aldehyde was added per assay.

Discussion

The biochemical lesions in the *aba2* and *aba3* mutants of *Arabidopsis thaliana* were identified by feeding potential ABA precursors to cell-free extracts and monitoring their conversion to ABA. Both the *aba2* and *aba3* mutants are impaired in the later steps of ABA biosynthesis.

The *aba2* mutant was unable to convert xanthoxin to ABA, but did convert ABA-aldehyde to ABA. Thus, *aba2* represents a novel mutant impaired in the ring modifications of xanthoxin to produce ABA-aldehyde. Chemical oxidation of the ring hydroxyl with pyridinium chlorochromate is sufficient for the quantitative conversion of xanthoxin to ABA-aldehyde (Schwartz and Zeevaart, unpublished results), indicating that the ring modifications results from a single enzymatic step and subsequent rearrangements. Therefore, a single enzyme converts xanthoxin to ABA-aldehyde and any additional mutants identified at this step should be allelic to *aba2*.

The previously identified *aba1* mutant in *Arabidopsis thaliana* and the newly isolated *aba3* mutant have pleiotropic phenotypes, which are in part independent of their ABA deficiency (Rock et al., 1991; unpublished observation). The *aba2* mutant may be useful for studying the physiological role of ABA, because the mutation appears to be specific for ABA biosynthesis.

The *aba3* mutant was unable to oxidize ABA-aldehyde to ABA. Lesions at this step may result from a defect in the aldehyde oxidase apoprotein or the Moco, which the aldehyde oxidase requires (Walker-Simmons et al., 1989; Leydecker et al., 1995).

Previously characterized eukaryotic Moco hydroxylases, such as xanthine dehydrogenase and aldehyde oxidase, require a desulfo moiety of the Moco (Wahl and Rajagopalan, 1982). Xanthine dehydrogenase and three aldehyde oxidase activities were absent in *aba3* extracts. The *aba3* mutant also accumulated large quantities of an unidentified, polar compound, which may be due to the loss of a Moco requiring enzyme. Nitrate reductase utilizes a variant form of the Moco, which does not contain a sulfur ligand, and its activity was unaffected in the *aba3* mutant. The activity of xanthine dehydrogenase in the *ma-1* mutant of *Drosophila* could be reconstituted by treatment of extracts with dithionite and Na_2S, which results in the addition of sulfur to the Moco (Wahl et al., 1982). The inactivation of WT Columbia extracts with CN^- and the subsequent re-activation with Na_2S suggests that ABA-aldehyde oxidase activity requires a desulfo moiety of the Moco. Treatment of extracts of *aba3* with dithionite and Na_2S resulted in the activation of ABA-aldehyde oxidase activity. Therefore, the genetic lesion in *aba3* appears to affect the introduction of a sulfur into the Moco (Figure 3.10).

The characterization of ABA-deficient mutants has been useful in elucidating the pathway of ABA biosynthesis. In *Arabidopsis* multiple alleles of *aba1* have been identified (Koornneef et al., 1982) and there are now two new mutants impaired in the later steps of ABA biosynthesis. No mutants have been reported in the cleavage reaction, which may be the key regulatory step in the pathway. Mutants impaired in the cleavage reaction may not be viable or the existence of multiple isozymes may make it difficult to identify these mutants. However, the apparent lability and low abundance of the cleavage enzyme make it unlikely that the activity can be purified by classical biochemical methods.

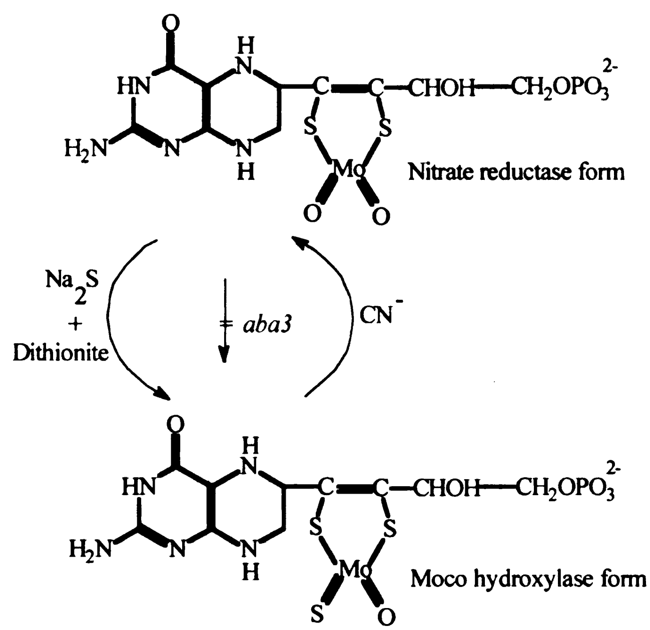


Figure 3.10- The chemical modification of the Moco and the proposed genetic lesion in the *aba3* mutant.

The search for additional mutants may be useful in the characterization of this step.

REFERENCES

Cornish K, Zeevaart JAD (1985) Abscisic acid metabolism in relation to water stress and leaf age in *Xanthium strumarium.* Plant Physiol **76:** 1029-1035

Creelman RA, Zeevaart JAD (1984) Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. Plant Physiol **75:** 166-169

Creelman RA, Gage DA, Stults JT, Zeevaart JAD (1987) Abscisic acid biosynthesis in leaves and roots of *Xanthium strumarium.* Plant Physiol **85:** 726-732

Duckham SC, Taylor IB, Linforth RST, Al-Naieb RJ, Marples BA, Bowman WR (1989) The metabolism of *cis*-ABA-aldehyde by the wilty potato, pea and *Arabidopsis thaliana.* J Exp Bot **40:** 901-905

Duckham SC, Linforth RST, Taylor IB (1991) Abscisic acid-deficient mutants at the *aba* gene locus of *Arabidopsis thaliana* are impaired in the epoxidation of zeaxanthin. Plant Cell Environ **14:** 601-606

Gaskin P, MacMillan J (1992) GC-MS of Gibberellins and Related compounds: Methodology and a Library of Reference Spectra. Cantock's Press, Bristol, UK

Koornneef M, Jorna ML, Brinkhorst-van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin-sensitive lines of *Arabidopsis thaliana* (L) Heynh. Theor Appl Genet **61:** 385-393

LaBrie ST, Wilkinson JQ, Tsat YF, Feldman KA, Crawford NM (1992) Identification of two tungstate-sensitive molybdenum cofactor mutants, *chl2* and *chl7*, of *Arabidopsis thaliana.* Mol Gen Genet **233:** 169-176

Léon-Kloosterziel KM, Alverez-Gil M, Ruijs GJ, Jacobsen SE, Olszewski NE, Schwartz SH, Zeevaart JAD, Koornneef M (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. Plant J **10:** 655-661

Leydecker M-T, Moreaux T, Kraepiel Y, Schnorr K, Caboche M (1995) Molybdenum cofactor mutants, specifically impaired in xanthine dehydrogenase activity and abscisic acid biosynthesis, simultaneously overexpress nitrate reductase. Plant Physiol **107:** 1427-1431

Linforth RST, Bowman WR, Griffin DA, Marples BA, Taylor IB (1987) 2-*trans*-

ABA-alcohol accumulation in the wilty tomato mutants *flacca* and *sitiens.* Plant Cell Environ **10:** 599-606

Parry AD, Neill SJ, Horgan R (1988) Xanthoxin levels and metabolism in the wild-type and wilty mutants of tomato. Planta **173:** 397-404

Rock CD, Zeevaart JAD (1991) The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci USA **88:** 7496-7499

Rock CD, Bowlby NR, Hoffman-Benning S, Zeevaart JAD (1991) The *aba* mutant of *Arabidopsis thaliana* (L.) Heynh. has reduced chlorophyll fluorescence yields and reduced thylakoid stacking. Plant Physiol **100:** 1796-1801

Sindhu RK, Walton DC (1987) The conversion of xanthoxin to abscisic acid by cell-free preparations from bean leaves. Plant Physiol **85:** 916-921

Taylor IB (1991) Genetics of ABA synthesis *In* Davies, WJ and Jones, HG (eds), Abscisic Acid, Physiology and Biochemistry. Bios Scientific, Oxford, UK, pp 23-25

Taylor IB, Linforth RST, Al-Naieb RJ, Bowman WR, Marples BA (1988) The wilty mutants *flacca* and *sitiens* are impaired in the oxidation of ABA-aldehyde to ABA. Plant Cell Environ **11:** 739-745

Taylor HF and Burden RS (1972) Xanthoxin, a recently discovered plant growth inhibitor. Proc R Soc London Ser B **180:** 317-346

Wahl RC, Warner CK, Finnerty V, Rajagopalan KV (1982) *Drosophila melanogaster ma-1* mutants are defective in the sulfuration of desulfo Mo hydroxylases. J Biol Chem **257:** 3958-3962

Wahl RC, Rajagopalan KV (1982) Evidence for the inorganic nature of the cyanolyzable sulfur in molybdenum hydroxylases. J Biol Chem **257:** 1354-1359

Walker-Simmons M , Kudrna DA, Warner RL (1989) Reduced accumulation of ABA during water stress in a molybdenum cofactor mutant of barley. Plant Physiol **90:** 728-733

Wray J, Filner P (1970) Structural and functional relationships of enzyme activities induced by nitrate in barley. Biochem J **119:** 715-725

Zeevaart JAD, Creelman RA (1988) Metabolism and physiology of abscisic acid. Annu Rev Plant Physiol Plant Mol Biol **39:** 439-473

Chapter 4

VP14 catalyzes the carotenoid cleavage reaction of abscisic acid biosynthesis.

ABSTRACT

The plant growth regulator abscisic acid (ABA) is formed by the oxidative cleavage of an epoxy-carotenoid. This is the first committed reaction in ABA biosynthesis and is believed to be a key regulatory step. The reaction is of general interest, because the synthesis of other apocarotenoids, such as vitamin A in animals, may occur by a similar mechanism. However, carotenoid cleavage reactions remain controversial, due to difficulties in demonstrating the activities *in vitro*. A new ABA-deficient mutant of maize has been identified and the corresponding gene, *VP14*, has been cloned. Here, it is shown that the recombinant VP14 protein catalyzes the cleavage of 9-*cis*-epoxy-carotenoids to form C_{25} apo-aldehydes and xanthoxin, a precursor of ABA in higher plants. This is the first carotenoid cleavage enzyme to be cloned and characterized from any organism.

INTRODUCTION

Apocarotenoids, compounds derived from the oxidative cleavage of carotenoids, are widely distributed in nature and have important metabolic and hormonal functions in diverse organisms. In Mucoraceous fungi, the hormone trisporic acid is a mediator of sexual processes (Bu'Lock, 1983). Retinal serves as a photosensory pigment in animals (Wald, 1968), green algae (Foster et al., 1984), and *Halobacterium* (Bibikov et al., 1993). Retinoids, vitamin A derivatives, are morphogens in animals (Giguere et al., 1987) and have clinical applications for cancer prevention and treatment (Hoffman, 1992; Cornic et al., 1994).

Apocarotenoids may be formed by random cleavage due to photo-oxidation or lipoxygenase co-oxidation. However, regulating the synthesis of biologically active apocarotenoids may require a more precise mechanism for their synthesis. Enzymatic cleavage of carotenoids at a specific position of the polyene chain has been proposed for the synthesis of several apocarotenoids. The most definitive illustration of enzymatic cleavage is the production of ß-cyclocitral (C_{10}) from ß-carotene by the cyanobacterium *Microcystis* (Jüttner and Höflacher, 1985). In other organisms, however, such enzymatic cleavage of carotenoids has been difficult to demonstrate *in vitro*. For this reason, the cleavage reaction in vitamin A biosynthesis remains controversial. Central cleavage of ß-carotene by a 15,15'-dioxygenase to produce two molecules of retinal has been reported (Goodman et al., 1967; Olson, 1993). However, there is also evidence for excentric cleavage of ß-carotene. Following cleavage, additional carbons are removed from the larger product, by a mechanism similar to ß-oxidation, to form one molecule of retinoic acid (Gerber and Simpson, 1990; Wang et al., 1992).

ABA is a plant growth regulator involved in the induction of seed dormancy and adaptation to various stresses, such as drought (Zeevaart and Creelman, 1988). A loss of cell turgor during desiccation results in the rapid synthesis of ABA. The elevated levels of ABA are, in part, responsible for stomatal closure, changes in gene expression, and other plant adaptations to stress.

Since the elucidation of the structure of ABA, a biosynthetic derivation from carotenoids has been proposed (Taylor and Smith, 1967). There is now biochemical and genetic evidence to support this hypothesis. Labeling experiments with $^{18}O_2$ suggest that ABA is synthesized from a large precursor pool, which already contains two of the four oxygens found in the molecule (Creelman and Zeevaart, 1984). Oxygen derived from a hydroxyl and epoxide of neoxanthin or violaxanthin (Figure 4.1) could account for the observed ^{18}O labeling pattern. In addition, when etiolated bean seedlings are stressed there is a decrease in the level of these xanthophylls and a corresponding increase in ABA and its catabolites (Li and Walton, 1990).

Direct evidence for a cleavage enzyme in ABA biosynthesis is lacking, due to the apparent low abundance and labile nature of the enzyme. Development of an *in vitro* assay has also been hindered by the presence of lipoxygenases and peroxidases, which oxidatively cleave carotenoids in a non-specific manner. Several features of the cleavage reaction have been inferred by analysis of the later steps in ABA biosynthesis (Figure 4.1). The initial C_{15} cleavage product, xanthoxin, is rapidly converted to ABA *in vivo* and *in vitro* (Sindhu and Walton, 1987). In cell-free extracts, *trans*-xanthoxin is converted to

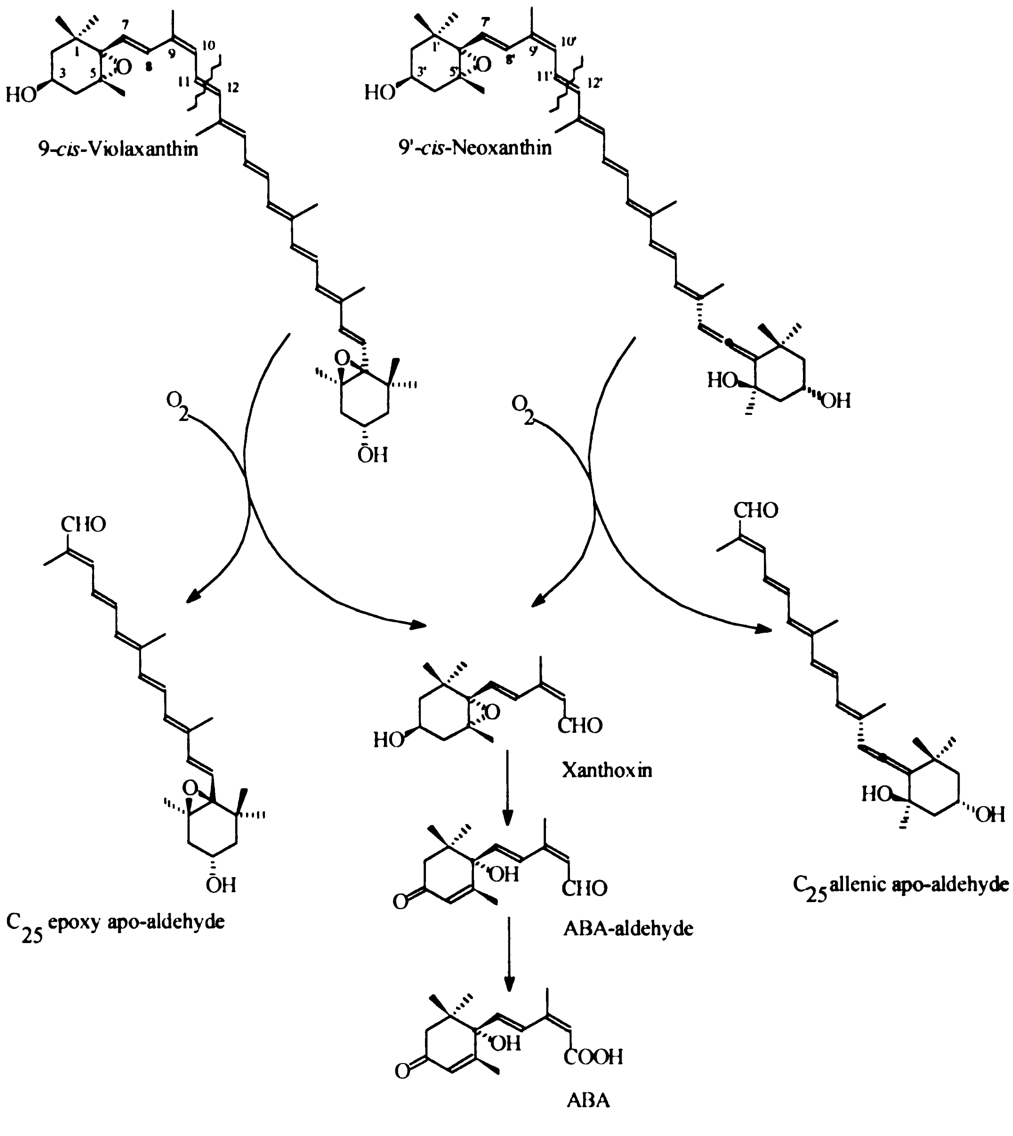


Figure 4.1- The proposed pathway of ABA biosynthesis in higher plants.

trans-ABA, indicating there is no *cis/trans* isomerization following cleavage (Sindhu and Walton, 1987). Thus, the xanthophyll precursor must have a 9-*cis* configuration to produce *cis*-xanthoxin and subsequently ABA, which is biologically active only in the *cis* form.

ABA biosynthetic mutants impaired at several steps in the pathway have been identified (Taylor, 1991). In maize, mutants with lesions in the early steps of carotenoid biosynthesis are ABA-deficient and have a viviparous phenotype. The *aba1* mutant in *Arabidopsis thaliana* (Rock and Zeevaart, 1991; Duckham et al., 1991) and the *aba2* mutant in *Nicotiana plumbaginifolia* (Marin et al., 1996) are impaired in the epoxidation of zeaxanthin and antheraxanthin to violaxanthin. A mutant in tomato, *notabilis*, may contain a lesion in the cleavage step (Parry et al., 1992). However, the difficulties of map-based cloning in tomato make this mutant of limited utility.

MATERIALS AND METHODS

Preparation of carotenoid substrates.

Spinach leaves were homogenized with a Waring blender in 100 mM phosphate buffer, pH 7.5. The homogenate was filtered through four layers of cheese cloth and centrifuged at 4,000 *g* to sediment the thylakoid membranes. Thylakoid membranes were saponified for three hours with a 10% solution of KOH (w/v) in methanol. Following saponification the carotenoids were partitioned into diethyl ether. The carotenoids were then resolved on a semiprep μPorasil HPLC column (30 x .078 cm) (Waters) eluted with a linear gradient of 10% ethyl acetate in hexane to 100% ethyl acetate over 72 min. The *aba1* mutant of *Arabidopsis thaliana* was used as a source for all-*trans*-zeaxanthin, which was isomerized with iodine (Zechmeister, 1962) and re-chromatographed to isolate 9-*cis*-zeaxanthin.

Enzyme assays.

VP14 was expressed as a GST-fusion protein. The recombinant protein was adsorbed to glutathione Sepharose and the VP14 protein was released by cleavage with thrombin according to the manufacturer's instructions (Pharmacia). Enzyme assays contained 100 mM Bis Tris buffer pH 6.7, 0.05% Triton X-100, 10 mM ascorbate , 5 μM $FeSO_4$, 1 mg/ml catalase, and recombinant VP14 protein. Assays were performed at 22-24°C in a total volume of 100 μL. The appropriate substrate was added in 3 μL ethanol. The enzyme assay and all subsequent procedures were performed under red light to minimize photo-oxidation of the precursors and products.

Identification of reaction products by mass spectromety.

The cleavage products were analyzed on a JEOL AX 505 double focusing mass spectrometer, equipped with a 5890 Hewlett Packard capillary gas chromatogram, using electron ionization at 70 eV. The trimethylsilyl derivative of xanthoxin was injected on a DB-5MS capillary column (J&W Scientific). The column was held at 100° C for 1 min., heated to 230° C at 40° C min^{-1}, and then at 8° C min^{-1} to 300° C. The C_{25} apo-aldehydes were introduced *via* a direct insertion probe heated from ambient temperature to 200°C at 64°C min^{-1}. The C_{15} cleavage product from zeaxanthin was analyzed as described above except that the probe was heated at 2° C min^{-1}. Exact mass measurements were performed as described above with the exception that the instrument's mass resolution was increased from 1000 to 7500. Perfluorokerosene was introduced simultaneously with the sample to provide reference ions to interpolate the exact mass assignments for sample ions.

Quantitative analysis of reaction products.

Following the incubation, 1 mL of H_20 was added to the reactions to dilute the detergent. The assays were then partitioned three times into an equal volume of ethyl acetate with vigorous stirring on a vortex-mixer. The ethyl acetate fractions were pooled, dried, and methyl ABA was added as an injection standard. The samples were stored under Ar at -80°C until analysis. Samples were injected on a µPorasil column (Waters) (30 x 0.4 cm) equilibrated with 90% hexane and 10% ethyl acetate. The column was eluted with a linear gradient to 20% hexane and 80% ethyl acetate over 15 minutes. A standard curve was constructed by injecting known quantities of the compounds analyzed and integrating the peak area. The integrated peak area of methyl-ABA was used to correct for variations in injections. The correlation coefficients were generally greater than 0.99.

RESULTS AND DISCUSSION

Characterization of the *vp14* mutant.

A new viviparous mutant in maize, *vp14,* has recently been identified (Tan et al., 1997). Viviparous mutants may result from a lesion in ABA biosynthesis or signal transduction (McCarty, 1996). Measurement of ABA levels in *vp14* embryos indicate that the mutant is impaired in ABA biosynthesis (Table 4.1). Homozygous *vp14* plants have a slightly wilty phenotype (Tan et al., 1997), which may also be accounted for by reduced ABA levels (Table 4.2).

There are some qualitative variations in the carotenoid composition of mutant embryos when compared to the wild type (Figure 4.2). Most notably, the level of

Table 4.1. ABA levels (ng/g FW), measured according to Leon-Kloosterziel et al., 1997, in embryos 16, 18, and 20 days after pollination (DAP).

DAP	WT sib	*vp14*-2274
16	83.3	23.3
18	127.5	36.1
20	71	43.7

Table 4.2- ABA levels (ng/g FW) in turgid and wilted leaves of the wild type and *vp14* mutant.

Treatment	NS-2274	*vp14*-2274
Turgid	7.3±3.5	9.6±2.0
Wilted	178.0±20.4	97.3±8.1

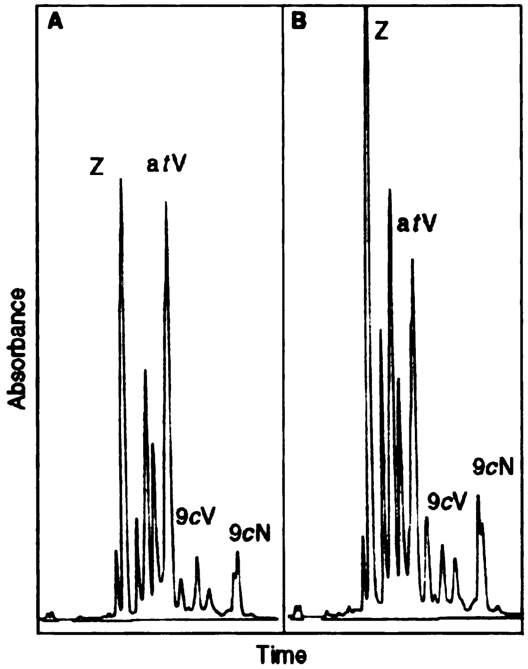


Figure 4.2- Carotenoid composition of WT and *vp14* embryos. 9-*cis*-violaxanthin (9*c*V), 9-*cis*-neoxanthin (9*c*N) and zeaxanthin (Z) are indicated on the chromatogram.

zeaxanthin is higher in wild type embryos. Zeaxanthin is the most abundant carotenoid in embryos, but is virtually undetectable in leaves. Therefore, decreases in zeaxanthin would be expected with precocious germination The efficient conversion of xanthoxin to ABA by cell-free extracts of the mutant (Table 4.3), indicates that the later steps in the pathway are not impaired in *vp14*.

The *in vitro* cleavage of carotenoids by recombinant VP14 protein.

The gene corresponding to the *vp14* mutant has been cloned (Tan et al., 1997) The derived amino acid sequence of VP14 (Tan et al., 1997) shows significant sequence similarity to lignostilbene dioxygenases (LSDs) from *Pseudomonas paucimobilis* (Kamoda and Saburi, 1993). The LSDs catalyze a reaction similar to the proposed cleavage reaction in ABA biosynthesis. Specifically, a double bond is oxidatively cleaved, yielding two products with aldehyde groups at the site of cleavage (Figure 4.3).

Using 9-*cis*-violaxanthin as substrate, the recombinant VP14 protein was tested for cleavage activity and the products were analyzed by HPLC (Figure 4.4). The expected cleavage products, xanthoxin and the C_{25} epoxy apo-aldehyde, were identified by their UV/VIS absorption spectra and mass spectra. The spectrum of xanthoxin was similiar to a previously reported spectrum (Gaskin and McMillan, 1992).

The spectra of the C_{25} allenic apo-aldehyde (3,5-dihydroxy-6,7-didehydro-12'-apo-ß-caroten-12'-al): *m/z* (relative intensity): 382 $[M]^+$ (100), 364 (20), 346 (18), 331 (9), 285 (27), 275 (7), 267 (22), 247 (18), 233 (67) 221 (24), 211 (50), 207 (36), 197 (43), 167 (83), 145 (71), 119 (33), 105 (68), 91 (86).

The spectra of the C_{25} epoxy apo-aldehyde (5,6-epoxy-3-hydroxy-12'-apo-ß-

Table 4.3- Conversion of xanthoxin to ABA by cell-free extracts of wild type (NS-2274) and *vp14* mutant. Assays contained 100 μg protein, NADP, EDTA,PMSF, and 100 ng xanthoxin.

Genotype	ABA
NS-2274	18.9± 2.1
vp14-2274	35.0± 1.4

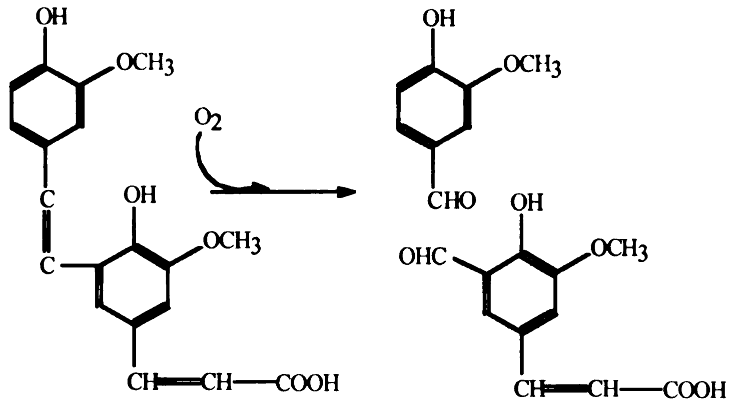


Figure 4.3- The reaction catalyzed by the lignostilbene dioxygenase (LSD) from *Pseudomonas paucimobilis* .

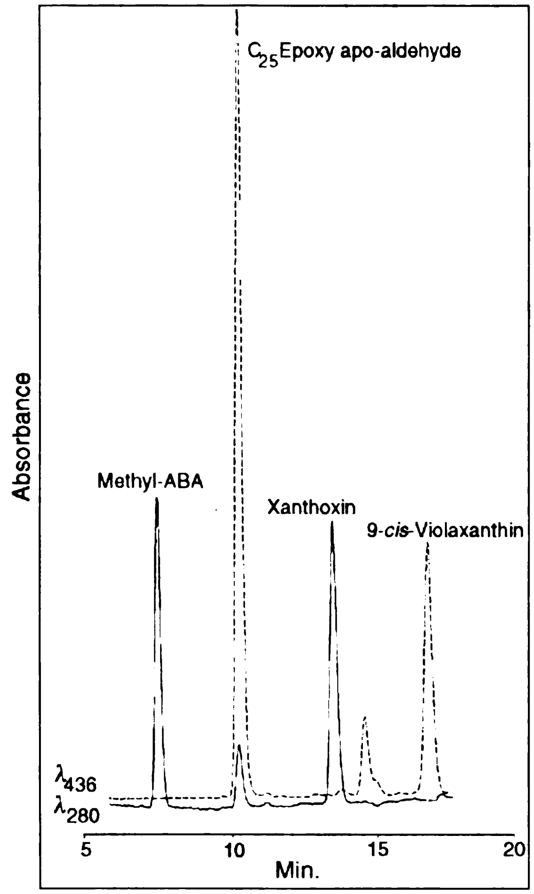


Figure 4.4- HPLC chromatogram of the cleavage reaction products using 9-*cis*-violaxanthin as substrate. Absorbance was measured at 436 nm and 285 nm with a photodiode array detector.

caroten-12'-al): *m/z* (relative intensity): 382 $[M]^+$ (100), 364(10), 302 (45), 287 (28), 234 (13), 221 (29), 207 (35), 173 (47), 159 (39), 145 (49), 135 (57), 119 (50), 105 (54), 91 (59).

The fragmentation patterns for the epoxy- and allenic- C_{25} cleavage products were nearly identical to published spectra for these compounds (Molnar and Szabolcs, 1979; Parry and Horgan, 1991). The theoretical mass of the isomeric C_{25} cleavage products from neoxanthin and violaxanthin ($C_{25}H_{34}O_3$) is 382.2508. The measured mass of the C_{25} product from neoxanthin was 382.2498 with an error of -2.6 ppm from the calculated mass. The measured mass of the C_{25} product from violaxanthin was 382.2501 with an error of -1.8 ppm.

Molecular oxygen (co-substrate), ferrous iron, and a detergent were all necessary for the cleavage activity (Table 4.4). The detergent was probably necessary for dispersion of the hydrophobic carotenoids. Catalase resulted in a slight increase in xanthoxin, possibly by reducing the non-enzymatic degradation of the substrate by a Fenton reaction (Chevion, 1988). A number of organic cofactors were initially added to the assays, but none had any effect on the activity (data not shown).

The cleavage reaction was totally inhibited by EDTA, a chelator of divalent cations (Table 4.5). Upon removal of the EDTA, the addition of equimolar concentrations of Fe^{2+} and the VP14 protein gave maximal activity; indicating tha the enzyme utilizes a single Fe^{2+} ion (data not shown). Because ferrous iron was necessary for activity, ascorbate was also added to the assays to maintain iron in the proper redox state. The divalent cations, such as addition of Ca^{2+}, Mn^{2+}, Mg^{2+} had no effect on the activity. Both $ZnCl_2$ and

Table 4.4- The requirements for cleavage activity *in vitro*. The standard reaction contained 0.05% Triton X-100, 5 μM $FeSO_4$, 10 mM ascorbate, and 1 mg/ml catalase in 100 mM Bis Tris buffer, pH 6.7 and 6 μg VP14 protein. Oxygen was eliminated by degassing the reactions under vacuum and purging with H_2 several times.

Triton X-100	Catalase	$FeSO_4$	O_2	Xanthoxin (ng)
+	+	+	+	865±10
-	+	+	+	n.d.
+	-	+	+	759±32
+	+	-	+	243±14
+	+	+	-	n.d.

Not detectable (n.d.)
ng xanthoxin ± SE, n=2

Table 4.5- The dependence of cleavage activity on ferrous iron. Iron was chelated from the VP14 protein with 50 mM EDTA. The EDTA was subsequently removed on a G-25 Sephadex spin column equilibrated with 100 mM Bis-Tris and 0.05% Triton-X 100. VP14 protein,7 μg, was then added to reactions containing the indicated cofactors.

Treatment	Xanthoxin (ng) ± SE, n=2
No iron	n.d.
5 μM Fe^{2+}	416.1±8.1
5 μM Fe^{3+}	n.d.
5 μM Fe^{3+}+10 mM ascorbate	432±8.2

$CuCl_2$ were inhibitory, presumably by competing with iron for binding to the active site.

With increasing VP14 concentration, there is a decrease in 9-*cis*-violaxanthin and an equimolar increase in xanthoxin and the C_{25} epoxy apo-aldehyde (Figure 4.5). Non-enzymatic cleavage resulting from photo-oxidation or Fenton chemistry would result in random cleavage at different double bond positions. However, the stoichiometric conversion of 9-*cis*-violaxanthin to the two products illustrates the specificity of the cleavage between the 11 and 12 positions of the polyene chain.

To determine the substrate specificity of the cleavage reaction, the all-*trans* and the 9-*cis* isomers of neoxanthin and violaxanthin were tested. The reaction products were separated on TLC plates and sprayed with 2,4-dinitrophenyl hydrazine to detect aldehydes (Figure 4.6). Xanthoxin and the predicted C_{25} products are present only in reactions containing the 9-*cis* isomers. The mono-epoxy carotenoids, antheraxanthin and lutein epoxide, are also potential precursors of ABA. In most plant tissues, these xanthophylls exist in the all-*trans* configuration (Parry et al., 1990) and, as expected, their all-*trans* isomers were not cleaved by VP14 (data not shown). The 9-*cis* isomer of zeaxanthin, formed by iodine isomerization of the all-*trans* zeaxanthin (Zechmeister, 1962), was cleaved at the 11 and 12 positions by the VP14 protein (Figure 4.6).

The spectra of the C_{25} zeaxanthin apo-aldehyde (3-hydroxy-12'-apo-ß-caroten-12'-al): *m/z* (rel. int.): 366 $[M]^+$ (100), 348 (6), 255 (4), 213 (8), 197 (8), 147 (17), 119 (20), 105 (15), 91 (15).

The theoretical mass of the cleavage product ($C_{25}H_{34}O_2$) is 366.2559 and the experimentally determined mass was 366.2564 with an error of 1.5 ppm from the

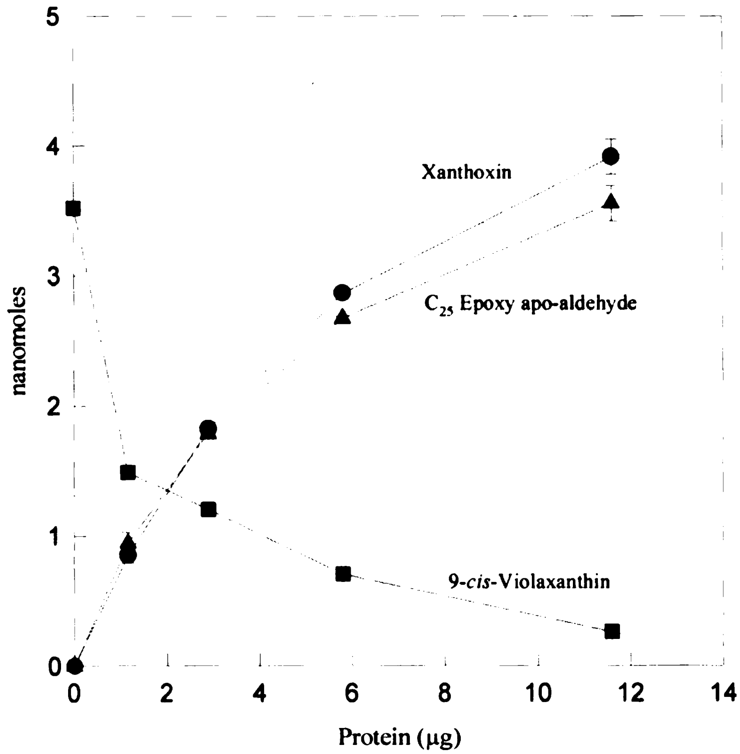


Figure 4.5- Decrease in 9-*cis*-violaxanthin and the concomitant increase in xanthoxin and the C_{25} apo-aldehyde as a function of the VP14 protein concentration. Assays were incubated for 10 min. at 22-24°C, extracted, and quantified.

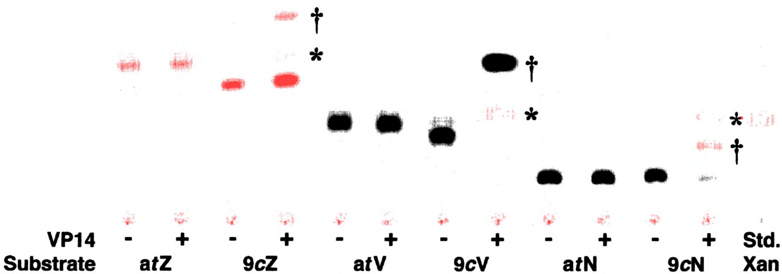


Figure 4.6- Thin-layer chromatography of assays with VP14 protein (+) and without (-). Assays contained approximately 5 µg of the indicated substrate: The 9-*cis* isomers (9*c*) and the All-*trans* isomers (a*t)* of Zeaxanthin (Z), Violaxanthin (V), and Neoxanthin (N). Substrate and products were separated on a silica gel 60 plate (EM Separations) developed with 10% *iso*-propanol in hexane. The plates were sprayed with 2,4-dinitro-phenylhydrazine to detect xanthoxin and other aldehydes. The C_{25} products are indicated by an (*) and the C_{15} products are indicated by an (†). The unlabeled spots are the carotenoid precursor.

calculated mass.

C_{15} zeaxanthin apo-aldehyde (3-hydroxy-apo-ß-caroten-11-al): *m/z* (rel. int.) 234 $[M]^+$ (67), 219 (17), 201 (48), 187 (35), 159 (34), 149 (79), 131 (43), 121 (52), 105 (52), 95 (100).

The compound ($C_{15}H_{22}O_2$) has a theoretical mass of 234.1620 and the experimentally determined mass was 234.1611 with an error of -3.7 ppm.

The cleavage of 9-*cis* zeaxanthin indicates that the 9-*cis* configuration is the primary determinant of cleavage specificity for the *in vitro* assays. Cleavage of 9-*cis*-epoxy carotenoids would result in the production of *cis*-xanthoxin, which would subsequently be converted to the biologically active isomer of ABA *in vivo*.

The environment of the carotenoids in the thylakoid and envelope membranes (Douce and Joyard, 1979) is very different from *in vitro* assays in which the carotenoid substrates are solubilized by detergent. However, the characteristics of the cleavage reaction, both in the substrate specificity and the position of cleavage, are consistent with the proposed pathway (Parry, 1993). Current evidence suggests this cleavage reaction is the key regulatory step in ABA biosynthesis (Parry, 1993). Further characterization of the cleavage reaction and its regulation may allow the manipulation of ABA levels *in planta*, which would affect such processes as seed dormancy, drought tolerance, and cold hardening.

The lignostilbene dioxygenases from *Pseudomonas* (Kamoda and Sburi, 1993) and VP14 comprise a novel class of dioxygenases that catalyze similar double bond cleavage reactions. The conserved sequences have also been identified in several plant ESTs, two

ORFs from the *Synechocystis* genome sequencing project (Kaneko et al., 1996), and a protein expressed in the retinal pigment epithilium of mammals, RPE65 (Hamel et al., 1993). The function of the gene products has not yet been determined. The expression pattern of the RPE gene is not consistent with a role in vitamin A biosynthesis, but it does set a precedent for this class of proteins in animals. Conserved sequences may be useful in identifying additional carotenoid cleavage enzymes necessary for the synthesis of other important apocarotenoids, such as vitamin A.

References

Bibikov SI, Grishanin RN, Kaulen AD, Marwan W, Oesterhelt D, Skulachev VP (1993) Bacteriorhodopsin is involved in halobacterial photoreception. Proc Natl Acad Sci USA **90:** 9446-94450

Bu'Lock JD (1983) Trisporic acids. *In* Biosynthesis of Isoprenoid Compounds. JW Porter & SL Spurgeon, Eds Vol **2:** 437-462 John Wiley & Sons New York, NY

Chevion M (1988) A site-specific mechanism for free radical induced biological damage: the essential role of redox-active transition metals. Free Radic Biol Med **5:** 27-37

Cornic M, Agadir A, Degos L, Chomienne C (1994) Retinoids and differentiation treatment: a strategy for treatment in cancer. Anticancer Res **14:** 2339-2346

Creelman RA, Zeevaart JAD (1984) Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. Plant Physiol 75: 166-169

Douce R, Joyard J (1979) Structure and function of the plastid envelope. Adv Bot Res **7:** 1-116

Duckham SC, Linforth RST, Taylor IB (1991) Abscisic-acid-deficient mutants at the *aba* gene locus of *Arabdiopsis thaliana* are impaired in the epoxidation of zeaxanthin. Plant Cell Environ **14:** 601-606

Foster KW, Saranak J, Patel N, Zarrilli G, Okabe M, Kline T, Nakanishsi K (1984) A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eukaryote *Chlamydomonas.* Nature **311:** 756-759

Gaskin P, MacMillan, J (1992) GC-MS of Gibberellins and Related Compounds:

Methodology and a Library of Reference Spectra. Cantock's Press, Bristol, U.K.

Gerber LE, Simpson KL (1990) Carotenoid cleavage: alternative pathways. Methods Enzymol **189:** 433-436

Giguere V, Ong ES, Segui P, Evans RM (1987) Identification of a receptor for the morphogen retinoic acid. Nature **330:** 624-629

Goodman DS, Huang HS, Kanai M, Shiratori T (1967) The conversion of all-*trans*-ß-carotene into retinal. J Biol Chem **241:** 1929-1932

Hamel CP, Tsilou E, Pfeffer A, Hooks JJ, Detrick B, Redmond TM (1993) molecular cloning and expression of RPE65, a novel retinal pigment epithilium-specific microsomal protein that is post-transcriptionally regulated in vitro. J Biol Chem **268:** 15751-15757

Hoffman SL (1992) Retinoids—"differentiation agents" for cancer treatment and prevention. Am J Med Sci **304:** 202-213

Jüttner F, Höflacher B (1985) Evidence of ß-carotene 7,8 (7',8') oxygenase (ß-cyclocitral, crocetindial generating) in *Microcystis.* Arch Microbiol **14:** 337-343

Kamoda S, Saburi Y (1993) Cloning, expression, and sequence analysis of a lignostilbene-α,ß-dioxygenase gene from *Pseudomonas paucimobilis* TMY 1009. Biosc Biotech Biochem. **57:** 926-930

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp strain PCC6803. II Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res **3:** 185-209

Li Y, Walton DC (1990) Violaxanthin is an abscisic acid precursor in water-stressed dark-grown bean leaves. Plant Physiol **92:** 551-559

Marin E, Nussaume L, Quesada A, Gonneau M, Scotta B, Hugueney P, Frey A, Marion-Poll A (1996) Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia,* a gene involved in abscisic acid biosynthesis and corresponding to the *ABA* locus in *Arabidopsis thaliana.* EMBO J **15:** 2331-2342

Molnar P, Szabolcs J (1979) Alkaline permanganate oxidation of carotenoid epoxides and furanoids. Acta Chim Acad Sci Hung **99:** 155-173

Parry AD, Horgan R (1991) Carotenoid metabolism and the biosynthesis of abscisic acid. Phytochemistry **30:** 815-821

Parry AD, Griffiths A, Horgan R (1992) Abscisic acid biosynthesis in roots. II. The effects of water stress in wild-type and an abscisic acid-deficient mutant (*notabilis)* plant of *Lycopersicon esculentum* Mill. Planta **187:** 192-197

Parry AD, Babiano MJ, Horgan R (1990) The role of *cis*-carotenoids in abscisic acid biosynthesis. Planta **182:** 118-128

Parry, AD (1992) Abscisic Acid Metabolism. In: Methods in Plant Biochemistry, Vol. 9, ch. 15, P.J. Lea, ed. pp. 381-402. Academic, NY

Rock CD, Zeevaart JAD (1991) The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. Proc Natl Acad Sci USA **88:** 7496-7499

Sindhu RK, Walton DC (1987) The conversion of xanthoxin to abscisic acid by cell-free preparations from bean leaves. Plant Physiol **85:** 916-921

Taylor IB (1991) Genetics of ABA synthesis. *In* Davies, WJ and Jones, HG (eds), Abscisic Acid, Physiology and Biochemistry. Bios Scientific, Oxford, UK, pp 23-25

Taylor HF, Burden RS (1972) Xanthoxin, a recently discovered plant growth inhibitor. Proc R Soc Lond Ser B **180:** 317-346

Taylor HF, Smith TA (1967) Production of plant growth inhibitors from xanthophylls: a possible source of dormin. Nature **215:** 1513-1514

Wald G (1968) Molecular basis of visual excitation. Science **162:** 230-239

Wang X, Krinsky NI, Tang G, Russell RM (1992) Retinoic acid can be produced from the excentric cleavage of ß-carotene in human intestinal mucosa. Arch Biochem Biophys **293:** 298-304

Zechmeister L (1962) Cis-Trans Isomeric Carotenoids, Vitamin A, and Arylpolyenes. Academic Press Inc. Publishers. New York pg. 85

Zeevaart JAD, Creelman RA (1988) Metabolism and physiology of abscisic acid. Annu Rev Plant Physiol Plant Mol Biol **39:** 439-473

Chapter 5

Future directions

Osmotic stress responses in *Anabaena* PCC 7120.

The focus of the current work was to study the function and regulation of salt-induced genes in the cyanobacterium, *Anabaena* sp. PCC 7120. Although the function of individual genes has not been determined, the properties of the SA6 regulatory mutant indicate that salt-induced proteins may have a role in stress tolerance. If so, it may be possible to increase salt-tolerance in *Anabaena* by cloning and over-expressing salt-induced transcripts. It may also be possible to increase the expression of multiple proteins simultaneously. A number of mutations have been identified in response regulators, which result in hyperactivity or constitutive expression of genes (summarized in Volz, 1993). A similar mutation in the SA6 response regulator might result in constitutive expression of multiple salt-induced transcripts. The AB5 plasmid rescue contains the *lux*AB genes under the control of promoter elements recognized by the SA6 response regulator. Therefore, it may be possible to identify mutations that lead to constitutive expression of the luciferase reporter in an *E. coli* strain containing the AB5 plasmid rescue and expressing the response regulator. A 1.7-kb DNA fragment containing the coding region for SA6 was cloned into pBluescript SK (Stratagene) and transformed into a mutator strain of *E. coli* (MU53). Plasmid DNA isolated from the mutator strain was used to transform *E. coli* DH10B containing the AB5 plasmid rescue. The transformants were screened for elevated luciferase expression. To date, no positive colonies have been identified in the screen.

In higher plants, signal transduction pathways similar to two-component regulatory systems are involved in the regulation of responses to ethylene (Chang et al., 1993) and cytokinin (Kamimoto, 1996). Additional genes with sequence similarity to two-component systems have been identified in the plant dbest sequence database. In *Sacchromyces cerevisae*, only one two-component regulatory system has been identified (Maeda et al., 1994). In *Neurospora crassa*, the *nik-1* encodes a two-component hybrid involved in hyphal development (Alex et al., 1996). The limited number of two-component systems in lower eukaryotes may indicate that the plant regulatory systems originated from endosymbiosis. In fact, an ORF with very high sequence similarity to the *etr* gene is present in the *Synechocystis* genome (Kaneko et al., 1996). The possibility that a homolog of the SA6 gene exists in plants has not yet been explored. Further clarification of the relationship between cyanobacterial and plant regulatory systems should be possible as the plant sequencing projects generate more data. The identification of similar sequences in cyanobacteria and plants, coupled with functional analysis in cyanobacteria, may provide some insight into the function and evolution of two-component systems in plants.

ABA biosynthesis.

Mutants have now been characterized in the first committed reaction of ABA biosynthesis and all subsequent steps (chapters 3 and 4). However, there are several interesting steps preceding the cleavage reaction. The epoxy-carotenoid precursor must have a 9-*cis* configuration for cleavage and subsequent conversion to ABA, but nothing is known about the mechanism of isomerization. If there is an isomerase that catalyzes this reaction, a loss of its activity would cause an ABA-deficient phenotype. The cellular location of the cleavage reaction has not been determined, but it has been suggested that the cleavage reaction occurs in the chloroplast envelopes, which contain small amounts of carotenoids. Carotenoids are synthesized in the thylakoid membrane and subsequently transported to the envelope; thus, a lesion in carotenoid transport to the envelopes may also result in ABA-deficiency.

In collaboration with the lab of Maarten Koornneef, the characterization of potential *aba* mutants in Arabidopsis has continued. Several mutants (DOR9, C3C, J45, J28, and A63) capable of germination on the gibberellin biosynthesis inhibitor, paclobutrazol, have been identified. In vegetative tissues, the mutants do not show reduced ABA levels, the conversion of xanthoxin to ABA is not impaired, and the carotenoid composition is normal (data not shown). The lesion in these mutants may be in a seed-specific ABA biosynthetic isozyme or the phenotype may not be associated with ABA biosynthesis.

Biochemical evidence suggests that the cleavage reaction is the key regulatory step in ABA biosynthesis (Parry, 1992). The induction of cleavage enzyme transcripts by dehydration of maize leaves (Tan et al., 1997) is consistent with this hypothesis. Future work should concentrate on testing this point directly by over-expressing the cleavage enzyme in transgenic plants. By altering ABA levels in transgenic plants, it may be possible to alter a number of physiological processes, such as cold hardiness, seed dormancy, and drought tolerance.

Mechanistic studies of the cleavage reaction catalyzed by VP14.

VP14 and lignostilbene dioxygenases comprise a novel class of dioxygenases, which oxidatively cleave double bonds. Additional genes with sequence similarity to this class of dioxygenases have been identified in the sequence databases, but their function has yet to be determined. Nothing is known about the reaction mechanism this class of enzyme catalyzes.

The all-*trans* carotenoids are not cleaved by the recombinant VP14 protein (chapter 4), nor do the all-*trans* isomers act as competitive inhibitors when included in enzyme assays with the 9-*cis* isomers (data not shown). Therefore, it appears that the VP14 protein is unable to bind the all-*trans* isomers. The binding characteristics of VP14 were explored in greater detail by testing additional carotenoid substrates[1] in the cleavage assay (Figure 5.1). The cleavage of 9-*cis* zeaxanthin indicates that the 5,6 ring epoxide is not necessary for activity. To determine the necessity of the ring hydroxyl, an attempt was made to test ß-carotene as a substrate. ß-Carotene, however, was insoluble in the reaction buffer; it was not cleaved by VP14. The cleavage of 9- and 9'-*cis* capsanthin, which contains a ß-ring and a κ-ring (five carbons), was also tested. In this reaction, the ß-ring C_{25} product was not observed indicating that VP14 cannot cleave adjacent to the κ-ring.

1

All *trans*-carotenoids, purified from a variety of sources, were isomerized with I_2 and the *cis/trans* isomers were separated by normal phase HPLC (see chapter 3). The 9-, 13-, and 15-*cis* isomers are the only ones that are sterically allowed (Zechmeister, 1962). Of these, the 9-*cis* isomer is usually the most abundant and the least polar, which makes it relatively easy to identify on HPLC chromatograms. Further confirmation of a 9-*cis* configuration was based on absorption spectrum. In particular, the 9-*cis* isomer has a small "*cis*-peak" (absorption peak near 340 nm) relative to the 13- and 15-*cis* isomers (Zechmeister, 1962).

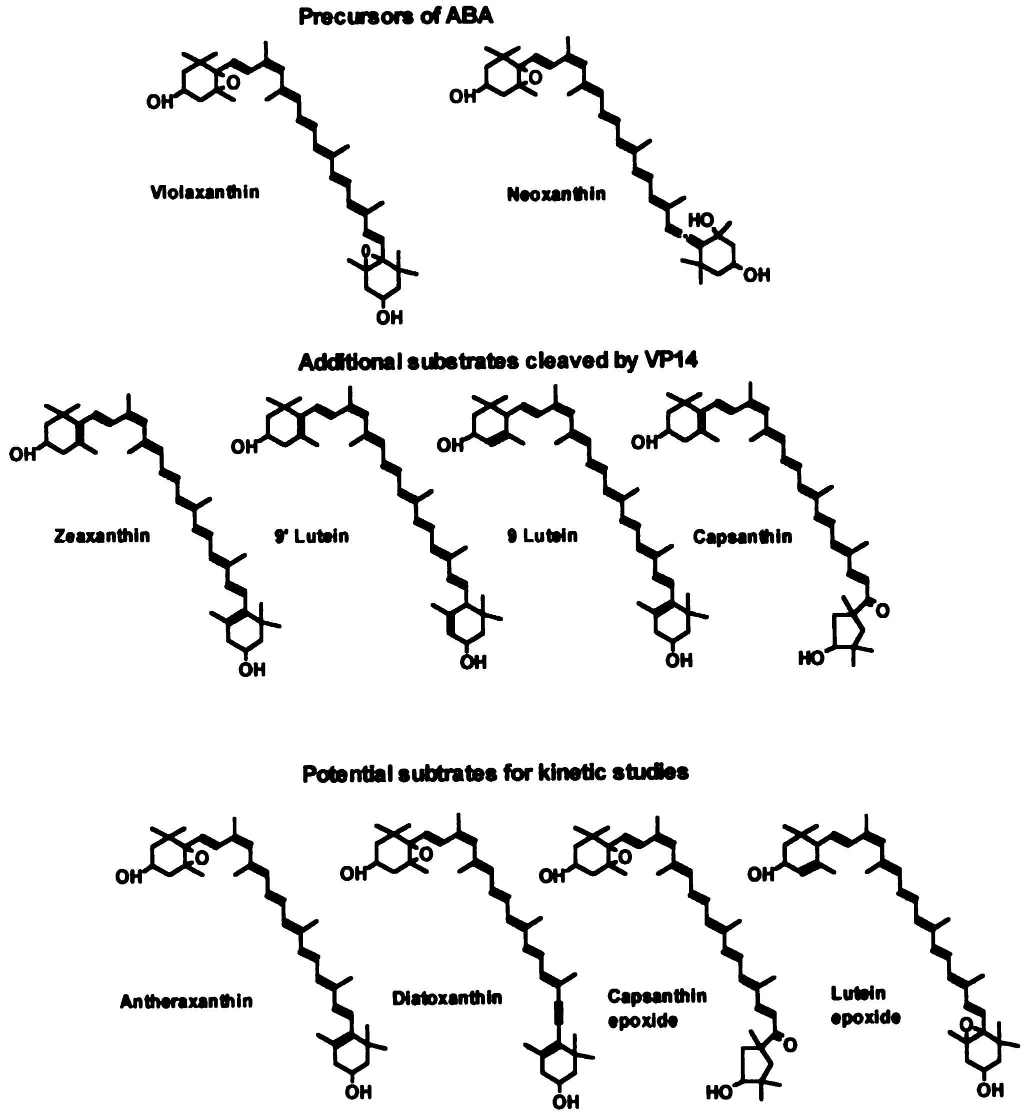


Figure 5.1- Cleavage substrates used in the characterization of VP14 activity.

Atmospheric oxygen concentrations are sufficient for maximal cleavage activity (data not shown). Therefore, the cleavage reaction should display pseudo-first order reaction kinetics with atmospheric concentrations of O_2. Kinetic measurements of the cleavage reaction with 9-*cis*-neoxanthin and 9-*cis*-violaxanthin showed similar Km value of approximately 10 μM (Figure 5.2), indicating that the enzyme binds the two substrates equally well. However, the Vmax for violaxanthin was significantly higher. At the site of cleavage, there are no structural variations to account for this difference. The electronic structure within the polyene chain may affect the rate of catalysis. The extent of photo- and chemical-oxidation of a carotenoid at different positions in the molecule is proportional to the electron density at a given position (Britton, 1995). By analogy, the rate of cleavage at the same position in different carotenoids may also be dependent upon the electron density. To determine if the rate of cleavage is proportional to electron density, semi-empirical molecular orbital modeling will be performed on violaxanthin and neoxanthin. The kinetic parameters for additional substrates will also be determined. A variety of asymmetric carotenoids exists in nature which contain a 5,6 epoxy and 4' hydroxyl group (Figure 5.1). Following isomerization to the 9- or 9'-*cis* configuration, these carotenoids should be cleaved by VP14 to produce xanthoxin. The structural variations at the other end of the molecule should affect the electron density in the polyene chain, so that the correlation with cleavage activity may be examined in greater detail.

Identification of inhibitors would also be beneficial in determining the reaction mechanism of VP14. There is a previous report that lipoxygenase inhibitors such as naproxen inhibit ABA biosynthesis (Creelman et al., 1992). At 10 mM, however, this

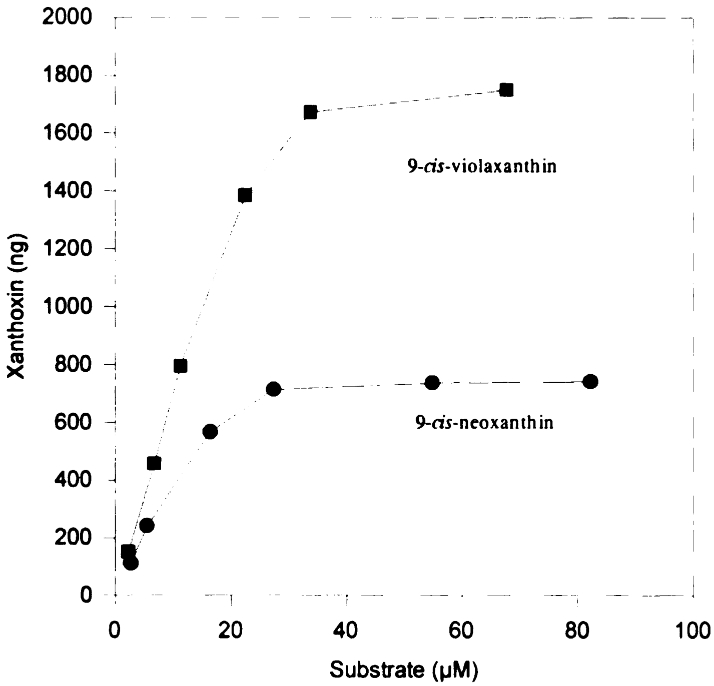


Figure 5.2- Kinetic measurements for the cleavage of 9-*cis*-violaxanthin and 9-*cis*-neoxanthin by VP14.

inhibitor had no affect on the cleavage activity (data not shown). To date, the only effective inhibitors are those which affect the binding of ferrous iron to the enzyme, such as iron chelators. Copper also inhibits the activity of VP14, presumably by competing with iron for binding to the active site (data not shown).

The nature of the iron coordination to the enzyme, determined by EPR spectroscopy, would also provide some insight into the reaction mechanism. While Fe^{2+} does not give a good EPR signal, it is possible to substitute Cu^{2+} in the active site. The inhibition of cleavage activity by Cu^{2+} (data not shown), indicates that this metal will compete with iron for binding to the active site.

REFERENCES

Alex LA, Borkovich KA, Simon MI (1996) Hyphal developement in *Neurospora crassa*: involvement of a two-component histidine kinase. Proc Natl Acad Sci USA **93:** 3416-3421

Britton G (1995) Structure and properties of carotenoids in relation to function. FASEB J **9:** 1551-1558

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science **262:** 539-544

Creelman RA, Bell E, Mullet JE (1992) Involvement of a lipoxygenase-like enzyme in abscisic acid biosynthesis. Plant Physiol **99:**1258-1260

Hansen G, Das A, Chilton MD (1994) Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium*. Proc Natl Acad Sci USA **91**: 7603-7607

Kakimoto T (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science **274:** 982-985

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki

A, Nakazaki N, Naruo K, Okumura S, Shimpo S,Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res **3:** 185-209

Maeda T, Wurgler-Murphy SM, Saito H (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369:** 242-245

Parry, AD (1993) Abscisic Acid Metabolism. In: Methods in Plant Biochemistry, Vol. 9, ch. 15, P.J. Lea, ed. pp. 381-402. Academic, New York

Tan BC, Schwartz SH, Zeevaart JAD, McCarty DR (1997) Submitted for publication.

Volz K (1993) Structural conservation in the CheY superfamily. Biochemistry **32:** 11741-11753

Wilde A, Churin Y, Schubert H, Borner T(1997) Disruption of a *Synechocystis* sp. PCC 6803 gene with partial similarity to phytochrome genes alters growth under changing light qualities. FEBS Lett **406:** 89-92

Zechmeister L (1962) Cis-Trans Isomeric Carotenoids, Vitamin A, and Arylpolyenes. Academic Press Inc. Publishers. New York pg. 85