AN ABSTRACT OF THE DISSERTATION OF

Pascale M. Williams for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on September 24, 1999. Title: Analysis of Coproporphyrinogen III Oxidase in Maize: the Genes, their Expression, and the Localization of their Products.

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Abstract approved: ____ _____

Carol J. Rivin

Coproporphyrinogen III oxidase (CPX) is an enzyme involved in the biosynthesis of tetrapyrroles, catalyzing the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX. In bacteria and yeast this enzyme is cytosolic, in animals it is mitochondrial, and in each it functions to produce heme. However, in all plants investigated, the initial steps of tetrapyrrole production, including those catalyzed by CPX, occur exclusively in the chloroplast stroma. Protoporphyrinogen IX is modified in the plastid envelope to produce chlorophyll and heme, or channeled to the mitochondria for heme production.

The maize genome has two CPX-encoding genes: CPX-1 has a putative amino-terminal chloroplast transit peptide, while CPX-2 has an amino-terminal sequence that is more consistent with mitochondrial transit peptides. Both genes are transcribed in all tissues tested but they have distinct expression patterns. The transcription level of *cpx-1* is greater than that of *cpx-2* in photosynthetic tissues. Neither gene shows light-dependent expression.

I have attempted to address the possibility that the two CPX-encoding genes have different roles in maize, and that their products have different cellular locations. One approach was the identification and characterization of *cpx* mutants to observe whether mutants of *cpx-1* and *cpx-2* have different phenotypes. A transposable element-disrupted *cpx-1* allele co-segregates with homozygous dek seed and yellow-lethal seedling phenotypes, consistent with a tetrapyrrole deficiency. In collaboration with Pioneer HiBred Intl., we identified additional *cpx* mutants. A plant with a *cpx-2* mutant allele appears to develop normally as a homozygote; *cpx-1* may complement this deficiency. Another approach was the immunological identification of CPX in protein preparations from isolated chloroplasts and mitochondria. CPX was detected in both the chloroplasts and mitochondria of wild-type plants; however, mitochondrial CPX was not detected in *cpx2-578* homozygotes. A third approach was to determine whether the amino-terminal sequence of CPX-2 would mobilize a fusion GFP protein into mitochondria. Maize epidermal cells expressing this construct have mitochondrial-sized foci of GFP localization with some general cytoplasmic background staining. The data supports the theory that CPX-1 is chloroplast-localized and participates in tetrapyrrole biosynthesis in that location. CPX-2 is the first mitochondrial-localized CPX enzyme identified in a plant and may function in tetrapyrrole production or the decontamination of excess coproporphyrinogen III.

Analysis of Coproporphyrinogen III Oxidase in Maize: the Genes, their Expression, and the Localization of their Products

by

Pascale M. Williams

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Pascale M. Williams, Author

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Dedication

I would like to dedicate this work to my uncle Tom Mulligan, who passed away during my studies in graduate school. He died before his time and had little opportunity to reach his potential. I hope to live with gratitude for the many opportunities life has offered me.

Analysis of Coproporphyrinogen III Oxidase in Maize: the Genes, their Expression, and the Localization of their Products

1. Introduction to Tetrapyrrole Biosynthesis and Regulation

1.1 Synopsis

This thesis describes my analysis of CPX in maize. Specifically, I will demonstrate the existence of two coprogen-encoding genes in maize and present evidence regarding the expression and localization of each enzyme. These data will be used to support my argument that each protein has a specific and individual function and to implicate their roles in tetrapyrrole biosynthesis. Chapter 2 contains the sequence information and analysis of the CPX-encoding genes. Chapter 3 addresses the transcriptional expression of *Cpx*. In chapter 4, I introduce the CPX mutants we have identified and a preliminary characterization of them. Evidence for the mitochondrial localization of the CPX2 enzyme is presented in chapter 5. And finally, chapter 6 is a summary of my findings and conclusions.

1.2 Introduction

Coproporphyrinogen III oxidase (coprogen) is an enzyme involved in the biosynthetic pathway of tetrapyrroles. Mutant analysis in yeast has determined that the expression of coprogen is critical to tetrapyrrole production (Bilinski *et al.* 1981). A coprogen null mutant does not survive without a tetrapyrrole supplement (Zagorec *et al.*

1988); thus, these molecules play a crucial role within an organism. The following section is a description of tetrapyrroles and their various functions.

A tetrapyrrole consists of an open or closed macrocycle of four pyrrole rings (figure 1.1). Features that are characteristic of specific families of tetrapyrrole molecules are the type of side-groups attached to the macrocycle and type of chelated metal within the porphyrin. Tetrapyrroles belong to the greater category of pigments, substances that absorb visible light (Campbell 1993). The structures of tetrapyrroles responsible for visible light absorption are the conjugated double-bond systems. The light absorption spectra is also determined by the functional groups bonded to the pyrrole rings and the presence of a chelated metal atom (Marks 1969). As a result, the absorption spectra of tetrapyrroles vary with their structure.

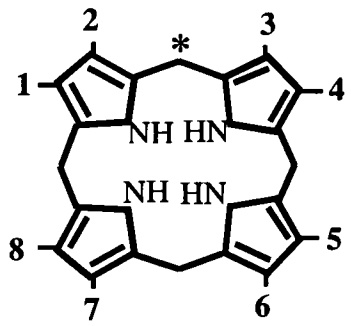

Figure 1.1 Fisher diagram of the basic structure of a porphyrin ring. Side chains that determine the physical characteristics of the porphyrin can be attached at positions 1 through 8. The asterisk represents the location of the successive oxidations during ring cleavage, resulting in the production of a linear tetrapyrrole.

Tetrapyrroles support a variety of critical functions in biological systems and are categorized by their general structure and function. Tetrapyrroles are generally associated

as the prosthetic group of an apoprotein, supporting enzyme catalysis (Marks 1969). The pyrrole ring functions as an electron carrier and is found in the form of heme in many cytochromes and oxidases, enzymes that transfer electrons to molecular oxygen. In specific circumstances, heme acts as an oxygen carrier, as in the case of hemoglobin and myoglobin. In addition, tetrapyrroles such as chlorophylls and phycobilines absorb light energy; this energy can be coupled to the high-energy chemical bond formation that takes place during photophosphorylation. Siroheme is the tetrapyrrole prosthetic group of nitrite and sulfite reductases. Corrinoids, such as vitamin B_{12}, compose another group of tetrapyrroles. An active derivative of vitamin B_{12}, 5' deoxyadenosyl cobalamin, is a coenzyme required in some carboxylation reactions. Coenzyme F430 is a tetrapyrrole found in methanogenic bacteria and is involved in the catalysis of methane formation. There are several families of tetrapyrrole molecules, and these families participate in a diverse set of reactions within an organism.

1.3 Heme and Chlorophyll

Two major types of tetrapyrroles are heme and chlorophyll. Heme is required by living organisms; it is central to all biological oxidations (Dailey 1990). Chlorophyll is essential to the light energy-harvesting reactions of photosynthesis (see figure 1.2). These molecules enable organisms, directly or indirectly, to utilize energy captured from light as chemical energy.

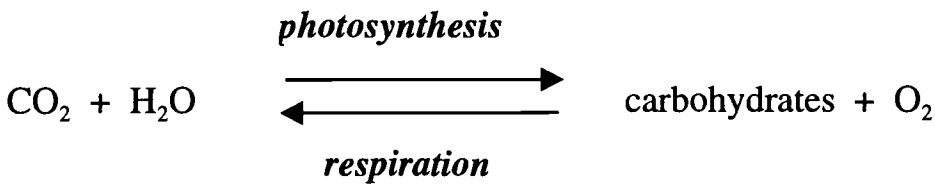

Figure 1.2 The relationship between photosynthesis and respiration. Adapted from Dailey, 1990.

1.3.1 Heme

There are three common types of heme found in both photosynthetic and non-photosynthetic organisms; these are heme *a* (figure 1.3), heme *b*, and heme *c* (Neuberger and Deenen 1991). These heme molecules all contain iron as the centrally chelated metal, but differ from one another by modifications of the side groups of the tetrapyrrole ring. In plants, heme *a* is the prosthetic group of mitochondrial cytochrome *c* oxidase. Heme *b* or protoheme is a component of respiratory cytochromes, the photosynthetic electron transport chain, peroxidases, plant microsomal cytochrome *P*-450, and other oxidative enzymes. Heme *c* is found in the cytochromes of the electron transport chain of both mitochondria and plastids.

One of the most critical roles of heme in biological systems is its function in the electron transport chain during cellular respiration. In eukaryotes, this process occurs in the inner membrane of the mitochondria. Electrons are removed from the high-energy bonds of NADH and $FADH_2$ and transferred to a series of electron carriers. This energy-releasing flow of electrons is coupled to the energetically unfavorable production of a

proton gradient across the mitochondrial membrane. The five cytochrome carriers of the electron transport chain are hemoproteins (Campbell 1993). There is also a heme-containing cytochrome complex that participates in analogous reactions of the photosynthetic electron transport chain. The ability of heme to act as an electron carrier facilitates crucial electrochemical reactions within the cell.

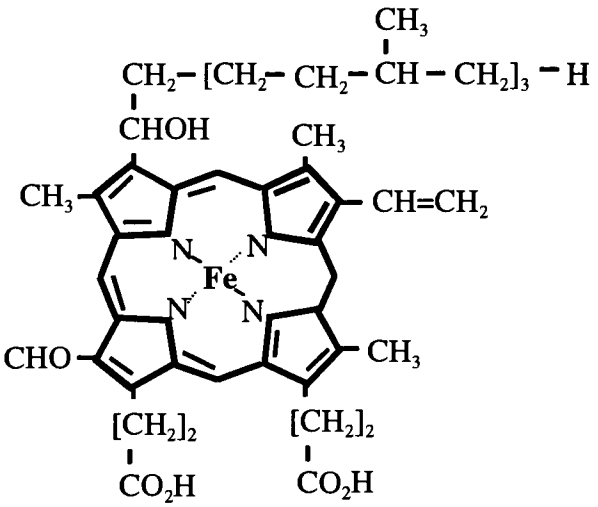

Figure 1.3 Heme *a* is shown as an example of the heme family of tetrapyrroles

Another important function of heme is its oxidative capacity. The hemoprotein cytochrome *P*-450 acts as a monooxygenase by catalyzing the hydroxylation of organic substrates, generally in the endoplasmic reticulum (Bolwell *et al.* 1994). Cytochrome *P*-450 has been associated with a broad range of secondary metabolic reactions in plants. The hemoproteins catalase and peroxidase both catalyze the breakdown of hydrogen peroxide, a by-product of the oxidative reactions that frequently take place in the

peroxisome (Ramasarma 1982). The oxidative reactions catalyzed by hemoproteins contribute to many different metabolic pathways.

Hemoproteins have been implicated in oxygen recognition and binding. Heme is the prosthetic group of hemoglobin and myoglobin, two well-characterized proteins that bind oxygen. Some plants express oxygen-binding leghomoglobins that are involved in the symbiotic interaction with rhizobial bacteria (Cutting and Schulman 1971). Leghemoglobin provides oxygen to the respiring bacteriods and may also sequester oxygen from the enzymes catalyzing nitrogen fixation. Recently, hemoglobin has been identified in plants (Hardison 1996) and is believed to facilitate oxygen transfer to respiring cells or act as a sensor of oxygen concentrations (Appleby *et al.* 1988).

There is evidence that heme itself plays a role in intracellular signalling (Padmanaban *et al.* 1989). Heme regulates its own biosynthesis (Reinbothe and Reinbothe 1995). For example, glutamyl-tRNA reductase, the second enzyme in the biosynthetic pathway of tetrapyrroles in plants, is inhibited by heme in barley (Pontoppidan and Kannangara 1994). Heme also acts at the level of transcription and translation. In yeast, the transcription of multiple genes is induced by the heme-activating protein complexes HAP1 and HAP2/3/4, whose DNA-binding properties are stimulated by heme (Zitomer and Lowry 1992). Heme has also been implicated in the control of protein synthesis in various mammalian systems (Marcus and Freedman 1986). Heme regulates the activity of a protein kinase in yeast known as the heme-controlled repressor (HCR) that phosphorylates initiation factor eIF-2; when heme is deficient, protein synthesis is inhibited (Kramer *et al.* 1976; Levin *et al.* 1976). Therefore, heme can affect gene expression at the levels of transcription, translation, and post-translationally.

Heme also plays a role in developmental regulation. Heme supplementation enhances the adipose differentiation of mouse 3T3-F442A fibroblast cells, while aminotriazole, a heme biosynthetic inhibitor, blocks differentiation. This block is overcome by hemin (Chen and London 1981). Erythroid differentiation is also stimulated by heme by initiating the transcription of multiple regulatory factors (Zhu *et al.* 1999). Both these examples utilize mammalian systems; heme has not yet been associated with a regulatory role in plant development. The hemoprotein complex cytochrome *P*-450 is involved in the biosynthesis of brassinosteroids, gibberellic acid, and abscisic acid; plant hormones that function to signal developmental changes. Therefore, heme does affect the production of plant hormones that regulate development. What is not known is whether heme controls these systems.

In summary, heme acts as an intracellular regulator in various organisms, affecting many cell systems. Heme plays a key role in respiration, oxidation, and oxygen transfer, reactions that are central in the functioning of living cells. Recently, it has been recognized that heme plays a more global role in cells, regulating multiple systems. Cellular heme levels may have a significant effect on the biochemical reactions that can occur in a cell and, in some mammalian systems, affect the developmental state of that cell.

1.3.2 Chlorophyll

The primary function of chlorophyll is as an electron carrier in the light reactions of photosynthesis. All chlorophyll molecules contain a centrally chelated magnesium atom and a fifth isocyclic ring (figure 1.4). There are three general chlorophylls found in

higher plants and algae, chlorophyll *a*, *b*, and *c*; there are also seven other chlorophylls found in bacterial systems, bacteriochlorophylls *a* through *g* (Neuberger and Deenen 1991). The chlorophyll molecules generally differ from one another in the oxidative state of their macrocyclic rings. Chlorophyll is a component of the light-harvesting complex, associated with the thylakoid membrane of the chloroplast. Photosystem I contains a higher ratio of chlorophyll *a* to chlorophyll *b*; photosystem II contains relatively more chlorophyll *b* and may also contain chlorophyll *c* (Lehninger 1982). Chlorophylls *a*, *b*, and *c* have defined roles in the process of photosynthesis. Chlorophyll *a* is the most abundant tetrapyrrole found in photosynthetic organisms (Dailey 1990). Chlorophyll *a* functions as both an accessory pigment of the light-harvesting complex, shuttling electrons within photosystems I and II, and as an electron donor at each reaction center. Chlorophyll *b* acts as an accessory pigment in both photosystems, contributing to the movement of electrons within each light-harvesting complex. Chlorophyll *c* is an accessory pigment found in many algae that do not contain chlorophyll *b* and acts as the accessory pigment in these systems. Each chlorophyll molecule has a specific function in photosynthesis.

Chlorophyll is a pigment essential to the process of photosynthesis. The capacity to photosynthesize enables primary producers to provide energy and biomass for non-photosynthetic organisms, and maintain oxygen levels in the biosphere. Therefore, the production of chlorophyll is critical to the survival of life on our planet.

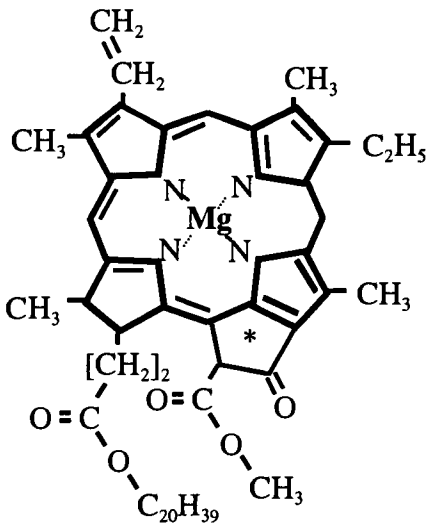

Figure 1.4 Chlorophyll *a* is shown here as an example of the chlorophyll family of tetrapyrroles. The fifth isocyclic ring characteristic of chlorophyll is marked with an asterisk.

1.4 Tetrapyrrole Biosynthesis

1.4.1 The Biosynthetic Pathway

In general, the basic pathway of tetrapyrrole biosynthesis is similar in all organisms (see figure 1.5), with species-specific variations in precursor formation and compartmentalization. Most of the enzymes and catalytic reactions are common to all organisms, but the initial reactions of the pathway in plants, algae, and some photosynthetic bacteria differ from that of animals, yeast, and other bacteria. The former category produces the first committed precursor, δ-aminolevulinic acid (ALA) from glutamic acid (Castelfranco and Jones 1975), the latter category produces ALA from succinyl CoA and glycine (Shemin and Rittenberg 1945; Shemin and Russell 1953). This difference correlates with the ability to photosynthesize. The first group of organisms produces both chlorophyll and heme, while the second group of organisms produces only

heme as their major end product, with the exception of some species of photosynthetic bacteria. The complete tetrapyrrole biosynthetic pathway of photosynthetic eukaryotes occurs in the chloroplast (Fuesler *et al.* 1984). In contrast, the tetrapyrrole biosynthetic pathway of animals and yeast are spread between the cytosol and the mitochondria (Dierks 1990; Labbe-Bois and Labbe 1990). The biosynthesis of tetrapyrroles is a well-conserved pathway.

In all versions of the biosynthetic pathway, the precursor ALA is produced (Reinbothe and Reinbothe 1996). Next, two molecules of ALA are condensed together to produce a five-membered ring, porphobilinogen. Four molecules of porphobilinogen are polymerized, resulting in the linear tetrapyrrole hydroxymethylbilane. Hydroxymethyl bilane is cyclized to produce uroporpyrinogen III, the universal precursor to all other tetrapyrroles. This cyclic tetrapyrrole is modified before insertion of a central chelated metal. Heme, chlorophyll, and phycobilins are produced from a subsequent intermediate, protoporphyrin IX. Siroheme and vitamin B_{12} are produced from the preceding intermediate, uroporphyrinogen III. All these tetrapyrroles are found in higher plants (figure 1.6).

Tetrapyrrole production is highly compartmentalized in plants; the tetrapyrrole biosynthetic pathway of is distributed between different locations within the chloroplast. All of the steps from ALA production to the formation of protoporphyrinogen IX occur within the stroma (Castelfranco *et al.* 1988; Nasri *et al.* 1988). The next enzyme, protoporphyrinogen IX oxidase is localized within the chloroplast envelope (Matringe *et al.* 1992). The final stages of chlorophyll biosynthesis, including activity of chlorophyll synthetase, occur in the thylakoid membrane (Rudiger *et al.* 1980). The enzymes of the

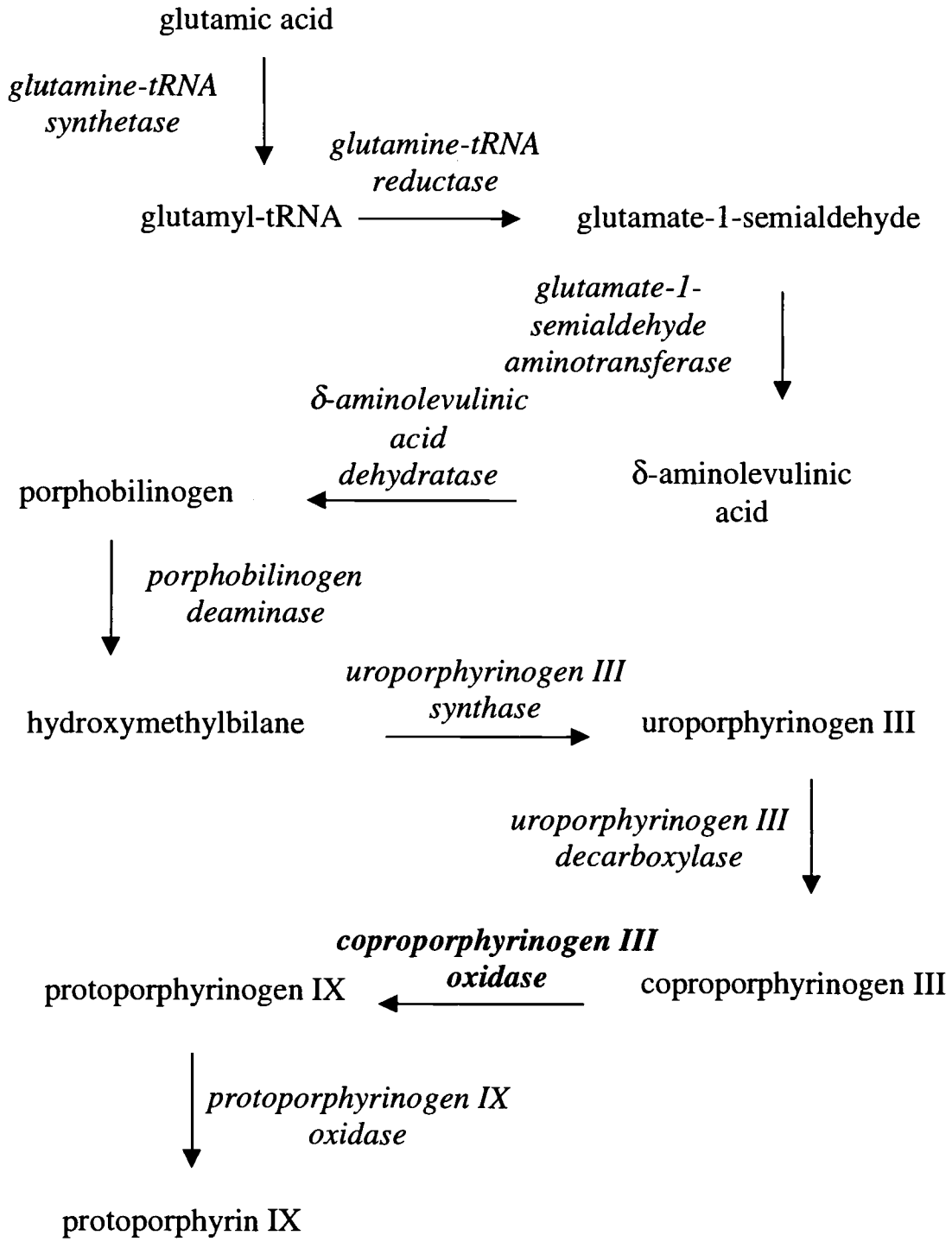

Figure 1.5 The tetrapyrrole biosynthetic pathway of angiosperms, from glutamic acid to the heme and chlorophyll precursor protoporphyrin IX. Adapted from Reinbothe and Reinbothe, 1996.

heme branch of tetrapyrrole biosynthesis are all located in the thylakoid membrane (Matringe *et al.* 1994). In addition, protoporphyrinogen IX oxidase and ferrochetalase activity has been associated with isolated mitochondria and plasma membrane (Jacobs and Jacobs 1995). The activity of the preceding enzyme, coprogen, was identified in chloroplasts and not in the mitochondria of pea (Smith, Marsh, and Elders 1993). It is believed that the chloroplast-generated product of coprogen, protoporphyrinogen IX is transferred from chloroplasts to mitochondria, providing a substrate for mitochondrial heme biosynthesis (Smith *et al.* 1993). Little is known about the transport of tetrapyrroles between compartments, proteins involved in this system have not been identified.

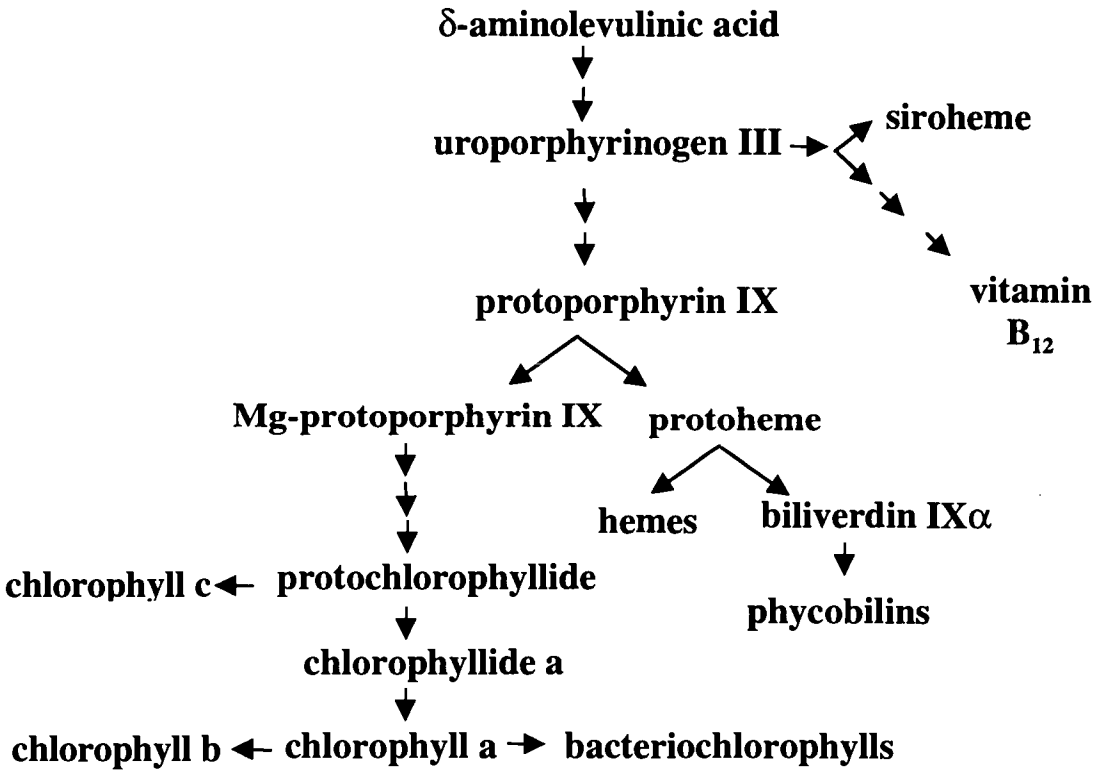

Figure 1.6 The biosynthetic relationship of tetrapyrroles characteristic of photosynthetic organisms. Adapted from Dailey, 1990.

1.4.2 The Regulation of Tetrapyrrole Biosynthesis in Plants

There are multiple mechanisms regulating the biosynthesis of tetrapyrroles (Reinbothe and Reinbothe 1996). The following is a summary of some of the major regulatory checkpoints that have been described.

First, the production of ALA is tightly regulated. Etiolated seedlings that are fed ALA in the dark accumulate protochlorophyllide and other Mg-porphyrin intermediates (Nadler and Granick 1970). Therefore, a critical stage in tetrapyrrole biosynthesis is ALA production, and the regulation can be bypassed by the addition of ALA. ALA synthesis is increased by the presence of light through what is believed to be a post-transcriptional mechanism (Reinbothe and Reinbothe 1996), promoting chlorophyll biosynthesis in photosynthetic tissue. Little is known about this regulatory step. Another factor in ALA production is the inhibition by heme of glutamine-tRNA reductase, an enzyme that participates in ALA synthesis. This mechanism provides a feedback control of the pathway. By controlling the production of ALA, the synthesis of tetrapyrroles can be limited.

Secondly, the insertion of a metal into the porphyrin ring is regulated. Magnesium chelatase inserts a magnesium ion into the porphyrin ring during the chlorophyll branch of tetrapyrrole biosynthesis. This process is inhibited in the dark. The transcription of a putative component of the magnesium chelatase complex, OLIVE (Gibson *et al.* 1995; Reinbothe and Reinbothe 1996) is downregulated in the light (Hudson *et al.* 1993). OLIVE is believed to bind protoporphyrin IX and prevent magnesium insertion by magnesium chelatase; thus, the presence of lower levels of OLIVE in the light would permit chlorophyll production and the higher levels of OLIVE in dark conditions would

favor heme production from protoporphyrin IX. Magnesium chelatase is also inhibited by protochlorophyllide; this feedback inhibition shuts down the chlorophyll branch of tetrapyrrole biosynthesis. Iron insertion into the porphyrin ring is also regulated. Ferrochelatase, the enzyme catalyzing iron insertion, is inhibited by heme, facilitating feedback inhibition of the heme branch of tetrapyrrole biosynthesis. By regulating the insertion of a metal ion into protoporphyrin IX, the balance between heme and chlorophyll production can be shifted.

Thirdly, chlorophyll is only produced from its precursors in the presence of light in some plants. Protochlorophyllide oxidoreductase (POR), the enzyme that converts protochlorophyllide *a* into the chlorophyll precursor chlorophyllide *a*, has two isomers, PORA and PORB. PORA is only active in the light and is subsequently rapidly degraded (Reinbothe *et al.* 1995) and its transcription is downregulated in the light (Forreiter *et al.* 1990; Holtorf *et al.* 1995). PORB is transcribed under both light and dark conditions, but its enzyme synthesis is low in the dark (Holtorf *et al.* 1995; Reinbothe *et al.* 1995). The isomers appear to play complementary roles in chlorophyll production and their activity requires light. Thus, tetrapyrrole biosynthesis is regulated at several stages and at multiple levels. The key checkpoints that control tetrapyrrole production are those that modulate ALA levels, influence magnesium or iron insertion into the porphyrin ring, and facilitate chlorophyll formation from protochlorophyllide.

1.5 Coproporphyrinogen III Oxidase

My studies are focused on the role of coproporphyrinogen III oxidase in maize. The following is a characterization of coprogen and its activity in other organisms.

Coprogen is the eighth enzyme of the tetrapyrrole biosynthetic pathway in plants (Dailey 1990). Coprogen functions to oxidize the proprionate groups at positions 2 and 4 on the porphyrin ring of coproporphyrinogen III to produce the vinyl groups of protoporphyrinogen IX (figure 1.7). Oxygen is the requisite electron acceptor and two molecules of CO_2 are released. In studies with coprogen purified from both bovine liver (Sano and Granick 1961; Yoshinaga and Sano 1980) and rat liver (Batlle *et al.* 1965), there was no cofactor associated with its activity. The bovine coprogen lost activity in the presence of tetranitromethane, indicating that at least one tyrosyl group is critical to activity (Yoshinaga and Sano 1980). The rat coprogen lost activity in the presence of a sulfhydryl reagent indicating the importance of a cysteine residue. Similar results have demonstrated that cysteine residues are essential for mouse and yeast coprogen activity. In *Saccharomyces cerevisciae*, coprogen has been determined to function as a homodimer; each subunit of the dimer contains one iron atom, but the iron has not been associated with enzyme activity (Camadro *et al.* 1986). The active site of coprogen has not been well characterized.

In previous studies, it was observed that the oxidation reactions of coprogen are successive, starting with the oxidation of the proprionate group at position II, resulting in a monovinyl 'harderoporphyrinogen' tricarboxyic intermediate (Elder *et al.* 1978; Jackson *et al.* 1980). Harderoporphyrinogen is believed to disassociate from the catalytic region and rotate within the active site, orienting the second proprionate group within the catalytic region. In a recent study characterizing the substrate and binding recognition of coprogen from chicken red cell hemolysates, it was determined that there is a lipophilic region near the catalytic site (Lash *et al.* 1999). This region participates in substrate

recognition and binding. The proprionate sidegroups of coproporphyrinogen III, at positions 6 and 7 of the porphyrin ring, are believed to associate with the lipophilic region and orient the porphyrin correctly within the active site. The model proposed by this analysis predicts that the harderoporphyrinogen intermediate must flip over and re-associate with the active site before the second oxidation reaction. The correct reorientation of harderoporphyrinogen requires the presence of these proprionate groups. Further studies are required in order to confirm this mechanism and to further characterize the active site of coprogen.

The expression of coprogen has been well-studied in yeast, where it is regulated by both heme and oxygen. Mutant strains of *Saccharomyces cerevisiae* deficient in the activity of δ-aminolevulinic acid, uroporphyrinogen decarboxylase, or ferrochelatase were all observed to have an increased level of coprogen transcripts and its protein product (Zagorec and Labbe-Bios 1986). Supplementation of heme decreases the transcription of coprogen in these mutants by a factor of 4-40x, depending on the background strain. Wild-type yeast showed a very similar magnitude of transcriptional down-regulation when exposed to aerobic conditions. It was concluded that both oxygen and heme negatively regulate coprogen expression. A subsequent study demonstrated that a region 5' from the coprogen-encoding gene was required for the de-repression of coprogen transcription during anaerobiosis (Zagorec *et al.* 1988). This region was associated with ROX1, a heme-controlled repressor, and HAP1-mediated regulation (Amillet *et al.* 1995). Under oxygen-limiting conditions, coprogen was characterized as the rate-limiting step of heme biosynthesis, likely the consequence of the oxygen-requirement in coprogen-catalyzed oxidations (Dailey 1990). Heme, itself, has been

described as an intermediate in the signalling mechanism for oxygen levels in yeast cells (Zitomer and Lowry 1992).

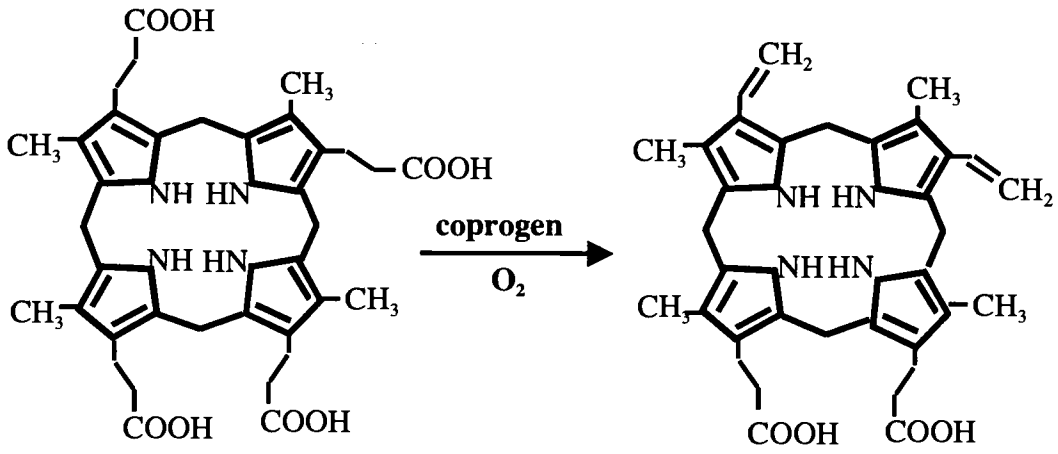

Figure 1.7 Coprogen catalyzes the oxidation of the carboxyl groups at positions 2 and 4 of the pyrrole ring.

The presence of a rate-limiting step in porphyrin biosynthesis can be determined by a profile of the porphyrin intermediates present. Ten strains of heterotrophic bacteria were analyzed for their porphyrin content, nine had coproporphyrinogen III as the significantly predominant porphyrin (Philipp-Dormston and Doss 1973). The tenth bacterial strain accumulated protoporphyrin IX and heme. In each of these strains, tetrapyrrole biosynthesis could be stimulated upon addition of ALA; therefore, δ-aminolevulinic acid synthetase regulated the overall synthesis of tetrapyrroles. However, a build-up of coproporphyrinogen III may indicate that coprogen is another limiting step in the pathway. Porphyrin profiles have also been performed on a couple of eukaryotic organisms. *Euglena gracilis* accumulates protoporphyrin IX under iron-limiting conditions (Carell and Price 1965; Karali and Price 1963) and not coproporphyrinogen

III, indicating a difference in the balance of porphyrins in this system. The leaves of *Nicotina tabacum* were analyzed for their porphyrin content under iron-limiting and non-limiting conditions. Under both conditions, coproporphyrinogen IX was the predominant porphyrin component in normal and chlorotic tissue (Hsu and Miller 1965). Coproporphyrinogen III is the predominant porphyrin in heterotrophic bacteria and tobacco leaves, but not in *Euglena* under iron-limiting conditions. The accumulation of coproporphyrinogen III may be correlated with a limitation in the activity of coprogen, an indication that coprogen catalyzes a rate-limiting step in tetrapyrrole biosynthesis in bacterial and plant systems.

The activity of a second coproporphyrinogen III oxidase enzyme has been detected in *Pseudomonas* (Ehteshamuddin 1968), *Chromatium* (Mori and Sano 1968), *Rhizobium japonicum* (Keithly and Nadler 1983), *Rhodobacter spheriodes* (Coomber *et al.* 1992; Seehra *et al.* 1983; Tait 1972), *Nitrococcus denitrificans* (Tait 1973), *Salmonella typhimurium,* (Xu *et al.* 1992), *Esherichia coli* (Troup *et al.* 1995), and *Saccharomyces cerevisiae* (Poulson and Polglase 1974). All of these enzymes can anaerobically oxidize coproporphyrinogen III using NADP or NAD as the electron acceptor and require the presence of various cofactors. Separate genes encode the aerobic and anaerobic versions of coprogen; there is very little homology between them (Coomber *et al.* 1992). Anerobic coprogen activity has not been observed in animals and higher plants. The presence of an anaerobic version of coprogen in bacteria and yeast may be a species-specific adaptation to anaerobic conditions.

In summary, studies of coprogen and its activity have characterized the enzyme from yeast and mammalian systems. If the active site of coprogen is conserved to that of

plants, catalysis may depend on a tyrosine and/or cysteine residue and substrate recognition and binding may require a hydrophobic region near the active site. One study in tobacco indicates that coprogen activity may be a rate-limiting step in tetrapyrrole biosynthesis. If coprogen activity is regulated, it may have a role in the regulation of tetrapyrrole production.

1.6 Mutations in the Tetrapyrrole Biosynthetic Pathway

There have been many documented cases of abnormal porhyrin production in humans as the result of mutations in tetrapyrrole biosynthetic enzymes (Dailey 1990). One set of abnormalities resulting from these mutations is a build-up of porphyrin intermediates and an increased solar photosensitivity. The porphyrin intermediates are phototoxic, generating oxygen free-radicals that cause cellular oxidative damage. In humans, this causes severe sunburning. Mutations in coprogen cause a syndrome known as coproporphyria.

In plants, a very similar effect results from a disruption in the expression of tetrapyrrole biosynthetic enzymes. Transgenic tobacco lines were generated that express antisense coprogen and protoporphyrinogen III oxidase transcripts (Kruse, Mock, and Grimm 1995b), disrupting the levels of the sense transcripts. These transgenic mutants build-up coproporphyrinogen III and protoporphyrinogen IX, respectively, and have differing severities of photodynamic damage, depending on the levels of the accumulated intermediate. In maize, a mutation was identified in uroporphyrinogen III decarboxylase, causing a build-up of the intermediate uroporphyrinogen III (Hu *et al.* 1998). The heterozygous phenotype of plants with this mutation is the development of light-

dependent necrotic lesions. Homozygotes produce yellow, necrotic seedlings that are detectably deficient for heme. Mutations in the biosynthetic pathway of tetrapyrroles cause light-dependent effect as the result of the accumulation of phototoxic tetrapyrrole intermediates.

1.7 Summary

Tetrapyrroles are ubiquitous molecules usually associated with enzymes and other proteins as prosthetic groups. Individual tetrapyrroles are associated with a metal cation and function as electron or oxygen carriers. Two major classes of tetrapyrroles, heme and chlorophyll, facilitate crucial cellular functions in respiration and photosynthesis. Coproporphyrinogen III oxidase is an enzyme involved in the biosynthesis of tetrapyrroles and has been studied in various organisms, including bacteria, yeast, mammals, and plants. In bacteria and yeast this enzyme is cytosolic, in mammals it is mitochondrial, and in all plants studied to date it is localized in the chloroplast. In this thesis I will describe my analysis of the coprogen-encoding genes in *Zea maize*, my conclusions regarding the activity of coprogen and its localization, and the overall significance of these conclusions in relation to tetrapyrrole biosynthesis.

2. Analysis of the CPX-Encoding Genes in Maize

2.1 Abstract

Coproporphyrinogen III oxidase (coprogen) is an enzyme of the tetrapyrrole biosynthetic pathway that catalyzes two oxidative decarboxylations of coproporphyrinogen III to produce protoporphyrinogen IX, a porphyrin precursor of both heme and chlorophyll. The higher plants studied to date have a single copy of the coprogen-encoding gene within their nuclear genome and the encoded product is transported to the chloroplast stroma. It is believed that the product of CPX, protoporphyrinogen IX, can move from the chloroplast to the mitochondria, supplying a precursor for mitochondrial heme synthesis. By homology, we have identified two coprogen-encoding genes in maize; *Cpx1* and *Cpx2*. Mapping and sequence comparison of these two genes demonstrates that they are highly homologous to one another and are likely to have resulted from a duplication event during the speciation of maize. The two genes encode almost identical coprogen enzymes, but they differ within the 5' region that encodes the presumptive organelle-targeting information. CPX1 has an amino-terminal region consistent with a chloroplast-targeting sequence; the amino-terminal region of CPX2 has the features of a mitochondrial-targeting peptide. Chloroplast-targeted CPX1 may function in the general production of tetrapyrroles in the chloroplast, including the creation of protoporphyrinogen IX for export to the mitochondria. The role of CPX2 in the mitochondria is hypothetical since the substrate molecule has not been reported in this location in other plants.

2.2 Introduction

Coproporphyrinogen III oxidase (coprogen) is the eighth enzyme in the biosynthetic pathway of tetrapyrroles in higher plants. The sequence of the gene encoding coprogen has been determined in many organisms, including bacteria, yeast, mammals, and several plants. Comparison of the amino acid sequences between these various species demonstrates 45-50% identity (Kruse *et al.* 1995). There are three highly invariant domains within the C-terminal region from all known coprogen enzymes. The active site of coprogen has not been defined; however, these regions clearly play an important role in the function of the enzyme. It has been shown that bovine coprogen has at least one tyrosine residue involved in its activity (Yoshinaga and Sano 1980), and there are six invariant tyrosine residues within the C-terminal region of all coprogen enzymes. Both plant and animal coprogen sequences have an amino-terminal extension that is not present in yeast (Zagorec *et al.* 1988) and bacteria (Xu and Elliott 1993; Troupe *et al.* 1994). These amino-terminal extensions are believed to be targeting sequences that direct the localization of coprogen an organelle, whereas, the yeast and bacterial enzymes are cytoplasmic.

Although there is very little homology between the amino-terminal regions of coprogen in barley, tobacco, and soybean, all are predicted to function as chloroplast targeting peptides. Features of the amino terminal extensions are consistent with that of a chloroplast transit sequence: these sequences have a high content of alanine and hydroxylated residues and a low content of acidic residues (Heijne *et al.* 1989). The putative cleavage sites of these sequences (Kruse, Mock, and Grimm 1995a) have a poor fit with the proposed cleavage site consensus (V/I)-X-(A/C)↓ A (Gavel and Heijne 1990).

However, in uptake experiments using intact pea chloroplasts, tobacco coprogen was imported into the stroma and detected as a mature, cleaved protein (Kruse, Mock, and Grimm 1995a). The homology between the plant coprogen enzymes begins with a conserved four amino acid motif 'IEKE' that occurs approximately 56 amino acids into each enzyme, immediately flanking the putative peptide cleavage sites, separating the targeting peptides from the mature proteins. The overall homology of coprogen from different plant species is 70% (Kruse, Mock and Grimm 1995a), with the relative level of homology increasing towards the C-terminal end of the sequence.

Two mammalian coprogen-encoding cDNAs, mouse and human, have been cloned. Sequence analysis and localization studies have identified mammalian coprogen as a mitochondrially-localized protein. Both of the encoded proteins have an amino-terminal extension of 31 residues. The mouse amino-terminal extension was reported to be a mitochondrial transit sequence based on its content of arginine and lysine residues, separated by hydrophobic residues (Kohno *et al.* 1993). Both the mouse and human amino-terminal extensions are rich in glutamic acid, which is unusual for a mitochondrial transit peptide (Heijne, Steppuhn and Herrmann 1989). However, localization studies support this prediction. Coprogen activity is associated within the intermembrane space of rat liver mitochondria, in a soluble form (Elder and Evans 1978). The mouse coprogen enzyme activity was identified in the soluble fraction of a mitochondrial extract (Kohno *et al.* 1993). Mammalian coprogen is a soluble enzyme localized within the mitochondria.

In mammals and in tobacco, soybean, and barley there is only one coprogen-encoding gene. As previously discussed, each enzyme is believed to be localized within a specific organelle. This information is consistent with what is known about tetrapyrrole

biosynthesis in general: it is highly compartmentalized, possibly in order to limit the oxygen radicals produced by the phototoxic pyrrolic intermediates to a location protected by antioxidants (Kruse, Mock, and Grimm 1995).

Maize is an organism with an interesting genetic history. As the result of an allotetraploid event, occurring approximately 11.4 million years ago, the entire genome was duplicated (Gaut and Doebley 1997). Since that time, the maize genome has undergone genetic rearrangements and losses and switched to disomic inheritance. The net result is a diploid organism with many duplicated loci (Rhoades 1951; Helentjaris, Weber, and Wright 1988). These loci are frequently arranged in clusters with the same gene order on different chromosomes and these are known as syntenous regions. A redundant genetic system provides opportunity for genetic drift without suffering the effect of a deleterious mutation in a critical gene. Gene pairs identified within these syntenous regions of maize have frequently diverged from one another, often specializing in their relative functions (Rhoades 1951; Wendel *et al.* 1989).

We have identified two coprogen-encoding genes in maize *(Cpx1* and *Cpx2)* that appear to have resulted from the allotetraploid genome duplication. I have compared these genes to coprogen-encoding genes of other organisms and identified putative targeting peptide sequences using computer-generated predictions. Based on this analysis, both maize genes encode the same enzyme, but CPX1 appears to be chloroplast-targeted, while CPX2 appears to be targeted to the mitochondria.

2.3 Methods

2.3.1 Library Screening for *Cpx1* and *Cpx2* Clones

A full-length genomic clone of the *Cpx1* gene was previously isolated and sequenced (Hardeman and Strom, unpublished data). From this clone, a 2.2 kb EcoR1/Sal1 fragment containing exons 5 through 8, pCPX2, was subcloned and used as a probe for subsequent library screening. The probe was radiolabelled using random priming (Feinberg and Vogelstein 1983) and diluted to a specific activity of 1.5 x 10^6 dpm/ml. This probe was used to screen a lambda-ZAP maize B-73 seedling leaf cDNA library (provided by Alice Barkan), following standard procedures (Sambrook *et al.* 1989). The blots were hybridized at 60° overnight in 5-10 ml of 250mM NaH_2PO_4, 7% SDS in a rotating hybridization oven (Robbins Scientific). The blots were washed once, briefly, with 2x SSC and three times with 0.5x SSC, 0.1% SDS, for 30 minutes each at 60°. The blots were exposed to autoradiographic film (Kodak) overnight with an intensifying screen (Dupont) or to a phosphoimager detection screen (Molecular Dynamics) for a few hours and analyzed using the ImageQuant program (Molecular Dynamics).

Four cDNA clones, pPW10, 11, 12, and 13, were identified and pBluescript was isolated from the phage. All four clones were sequenced by an automated sequencing service provided by the Central Services Laboratory (CSL) at Oregon State University. Each clone was determined to be a partial cDNA from a *Cpx1* transcript using Bestfit alignment software from the Genetics Computer Group (GCG, University of Wisconsin). The longest clone, pPW13, encoding exon 3 to the 3' UTR, was used for subsequent library screening.

A lambda-DASH II maize B-73 genomic library (provided by Pioneer HiBred International) was screened using pPW13 as a probe and a partial clone of *Cpx2* was identified that contains exons 2 through 5. Two DNA fragments were subcloned: a 3.6 kb EcoRI fragment (pPW235) and a 7.4 kb BglII fragment (pPW238). pPW238 was subcloned once again, yielding a 2.5 kb EcoRI/BglII fragment (pPW248). Each subclone was sequenced (CSL) and the sequences were merged using GCG software.

A lambda-MAX maize B-73 whole seedling cDNA library (CLONTECH Laboratories, Inc.) was screened using pPW13 as a probe and four more cDNA clones were identified, pPW239, 240, 241, and 244. The embedded bacterial/yeast shuttle vector, pYEUra3, was excised from each and sequenced (CSL) using primers flanking the cDNA insert. All of these cDNAs were determined by homology to be partial clones of *cpx-1* transcripts because the 3'UTR ends are nearly identical. However, there is a possibility that *Cpx1* and *Cpx2* are identical for the length of exons 6 through 8 and that some of these clones are partial *Cpx2* cDNAs. None of the isolated cDNAs were identified as full-length clones.

Exon 1 of *Cpx2* was determined by sequencing the genomic PCR products of *Cpx2* mutator insertional alleles *cpx2-104*, *cpx2-323*, and *cpx2-578*. A description of these mutants is included in chapter 4.

2.3.2 Sequence Analysis

DNA and protein sequences were compared using GCG Bestfit and aligned using GCG Pileup, Lineup, Pretty, and GrowTree. The sequences of other CPX-encoding genes were downloaded over the World Wide Web from GenBank

(http://www.ncbi.nlm.nih.gov/Entrez/). Organelle transit peptides were identified using PSORT Prediction of Protein Localization Sites, version 6.4 (http://psort.nibb.ac.jp:8800/form.html) (Nakai and Kanehisa 1992). Chloroplast transit peptides were identified using ChloroP, version 1.0 (http://cbs.dtu.dk/services/ChloroP/) (Emanuelsson *et al.* 1999).

2.4 Results

2.4.1 The Coprogen-Encoding Genes

Our lab has sequenced two coprogen-encoding genes in maize; the genes are designated *Cpx1* and *Cpx2*. *Cpx1* was assigned to map location 2S-036; *Cpx2* was mapped to 10L-116 (Hardeman *et al.* 1996). These map locations correlate with regions that have been previously described as syntenous for multiple loci (Moore *et al.* 1995). The sequence of *Cpx1* was determined from a genomic clone (Hardeman and Strom 1996); the coding sequence was predicted by homology to other plant CPX-encoding genes and verified by comparison to the partial cDNA clones (figure 2.1A). The first in-frame methionine codon is the presumed start codon; this methionine aligns with the start codons of barley, tobacco, and soybean (data not shown). *Cpx1* has eight exons, an exon/intron structure that is similar to that of the soybean gene (Madsen *et al.* 1993).

The partial sequence of *Cpx2* was determined for exons 1 to 5 from a partial genomic clone and from genomic PCR sequence (figure 2.1B) and was compared to the sequences of *Cpx1* and the soybean coprogen-encoding gene *(Cpo)*. The putative coding regions of *Cpx1* and *Cpx2* have an 84% identity (from exons 1 to 5, inclusive). This homology is based on the premise that the coding region of *Cpx2* begins at the first in-

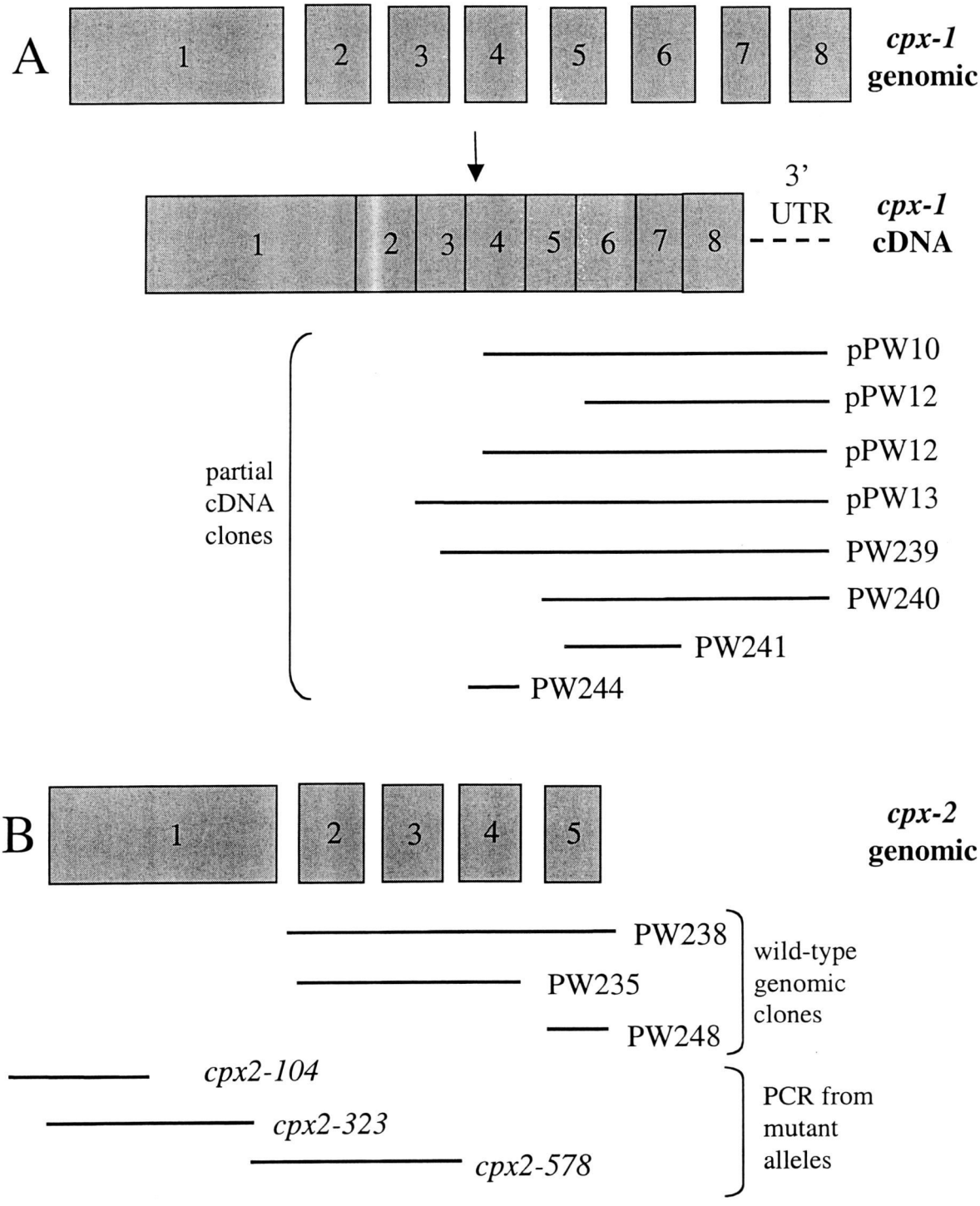

Figure 2.1 Sequence analysis of *Cpx*. The sequence of *Cpx1* was determined (A) and the coding region was verified by eight partial *Cpx1* cDNA clones. The sequence of *Cpx2* was determined (B) from one original genomic clone and its subclones, and from PCR sequence of *Cpx2* mutant alleles. The boxed regions represent open-reading frames and lines represent cloned or PCR-amplified regions.

frame methionine codon. (Evidence supporting this premise will be discussed in chapter 5). The intron/exon structure of *Cpx2* resembles that of *Cpx-1* and soybean *Cpo*. Significant deviations between the two *Cpx* genes occur in the 5' ends. There is a 150 nucleotide deletion in the *Cpx2* gene corresponding to bases 23-172 of the *Cpx1* gene (figure 2.2). In spite of this deletion, *Cpx2* has an in-frame methionine codon that aligns with the predicted methionine start codon of *Cpx1* (figure 2.3). 213 bases upstream from this codon is another in-frame methionine codon, this is the predicted start codon of the *Cpx2* gene. Apart from this variation in the 5' regions of *Cpx1* and *Cpx2*, the CPX-encoding genes of maize are 94% conserved at the DNA level.

2.4.2 The Translated CPX Products

The primary amino acid sequences of the maize CPX enzymes were deduced from a combination of cloned partial cDNAs, genomic PCR products, and genomic clones. The apparent size of the full-length CPX1 enzyme is 399 amino acids or 44.5 kDa. The CPX2 enzyme, translated from the partial sequence of *Cpx2* from the first in-frame methionine codon through exon 5, is 322 amino acids and 35.6 kDa (as compared to 302 amino acids of CPX1 for this region). The homology of the two CPX products is 95% within the putative catalytic region. If the C-terminal region of CPX2 is identical to that of CPX1, then the predicted full-length CPX2 enzyme, from the upstream methionine to the end of exon 8, is expected to be 419 amino acids and 47.0 kDa.

The maize CPX enzymes exhibit 80% amino acid identity. This is higher than the homology either between maize CPX1 and coprogen of its close relative barley (79% identity) or between maize CPX2 and barley (75% identity, only the translated sequences

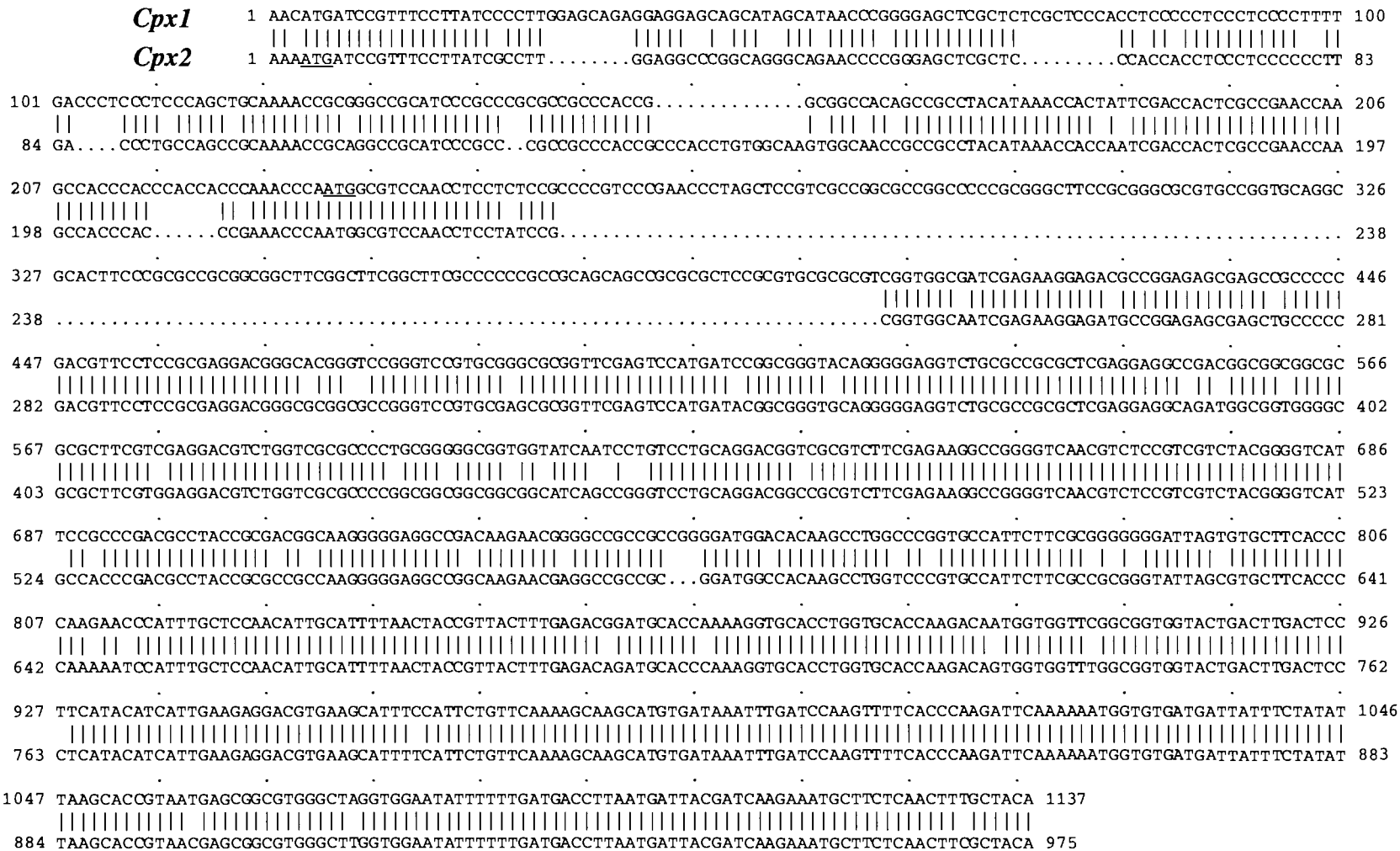

Figure 2.2 Alignment of the coding regions of *Cpx1* and *Cpx2*. The putative start codon for each gene is underlined.

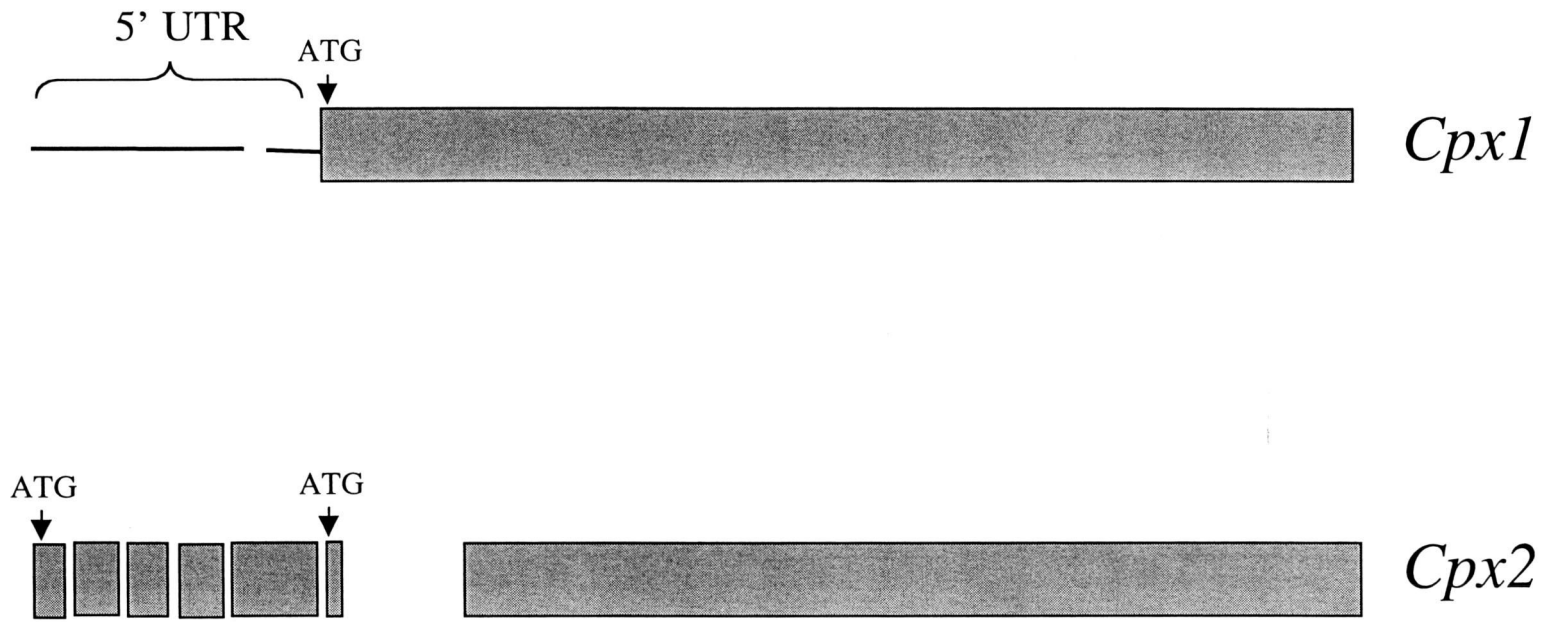

Figure 2.3 Schematic of *Cpx1* and *Cpx2* DNA alignment. The grey boxes represent contiguous coding regions, the spaces represent deletions compared to the other *Cpx* genomic sequence, and the 5' untranslated region (5'UTR) is represented by a line.

of exons 1-5 were compared). The homology between all known plant CPX enzymes is demonstrated by an amino acid alignment (figure 2.4).

The similarity between CPX enzymes of maize and that of 9 other species is displayed in a graphical format (figure 2.5). Only the amino-terminal portion of these enzymes were used in this study (the equivalent of amino acids 1-189 of CPX1 of maize) because this region is much less conserved between species. The distances represented in this dendogram are not proportional to the relative distances; however, they are grouped according to their relative homology. The level of conservation between the maize CPX enzymes is consistent with their predicted evolutionary relationship. Both CPX1 and CPX2 are conserved in three highly invariant domains, as described by Kruse *et al* (Kruse, Mock and Grimm 1995a). The three conserved domains are located between amino acids 222-244, 259-288, and 335-368 of the CPX1 sequence (figure 2.4). The only significant variation between both CPX1 and CPX2 of maize and other coprogen enzymes is the presence of a histidine residue at position 259 of CPX1, in place of a conserved tyrosine residue. In keeping with other coprogen enzymes, the level of homology is higher in the C-terminal region.

Both CPX1 and CPX2 are predicted to have an amino-terminal extension in relation to that of bacterial and yeast coprogen enzymes. Although they are not well conserved, these amino-terminal extensions are consistent with the coprogen enzymes of other plants and mammals and are likely to function as organellar-targeting sequences. To analyze the possible targeting information in the CPX1 and CPX2 proteins, I used two computer programs that look at conserved features between targeting peptides. This program analyzes the residue composition and overall charge of the putative transit

```
                1                                                  51                                                 100
maize CPX1      ..........  ..........  ..........  ..........  ..........  ..........  ..........  .MASNLLSAP  SRTLAPSPAP  APAGFRGRVP
maize CPX2      MIRFLIALEA  RQGRTPGARS  HHLPPPLTLP  AAKPQAASRP  PPTAHLWQVA  TAAYINHQST  TRRTKPPTRN  PMASNLLSA.  ..........  ..........
barley          ..........  ..........  ..........  ..........  ..........  ..........  ..........  .MASSLLTTP  SQTLAPNPAA  ARARRSSPAA
soybean         ..........  ..........  ..........  ..........  ..........  ..........  ..........  .M....MHCA  SIVSAPSYAF  P...FRSGSA
tobacco         ..........  ..........  ..........  ..........  ..........  ..........  ..........  .MLTPILSSA  SCSWTPTSQF  PHSWHSSPSF
consensus       ..........  ..........  ..........  ..........  ..........  ..........  ..........  .M.........  ..........  ..........

                101                                                151                                                200
maize CPX1      VQAHFPRRGG  FGFGFAPRRS  SRALRVRASV  AIEKETPESE  PPPTFLRE.D  ..G...HGSG  SVRARFESMI  RRVQGEVCAA  LEEADGGGAR  FVEDVWSRPC
maize CPX2      ..........  ..........  .........V  AIEKEMPESE  LPPTFLRE.D  ..G...RGAG  SVRARFESMI  RRVQGEVCAA  LEEADGGGAR  FVEDVWSRPG
barley          AQ......VS  FSSPLLPGR.  .RALRCARPV  AIEKEVPEKE  APTTFLRE.D  GSG...AGSG  SVRERFEGMI  RRVQGEICAA  LEEADGSGKR  FVEDVWSRPG
soybean         STTPTAISLT  KRS.WKPPPS  MAKGPVRATV  SIEKETPEAN  RPETFLRGVD  EA....QSST  SVRARFEKMI  REAQDTVCSA  LEAADG.GAQ  FKEDVWSRPG
tobacco         LTKPLNLPFT  ESY.KTAKRP  TPNYSFKVQA  MIEKEVAVSH  KPDAFLRESD  MGSNVTSNSS  SVRGRFEKMR  REAQDSVCLA  IEKADG.GAK  FKEDVWSRPG
consensus       ..........  ..........  ..........  .IEKE.....  .P..FLR..D  ..........  SVR.RFE.M.  R..Q...C.A  .E.ADG.G..  F.EDVWSRP.

                201                                                251                                                300
maize CPX1      GGGGINPVLQ  DGRVFEKAGV  NVSVVYGVIP  PDAYRDGKGE  ADKNGAAAGD  GHKPGPVPFF  AGGI.SVLHP  KNPFAPTLHF  NYRYFETDAP  KGAPGAPRQW
maize CPX2      GGGGISRVLQ  DGRVFEKAGV  NVSVVYGVMP  PDAYRAAKGE  AGKNEAAA.D  GHKPGPVPFF  AAGI.SVLHP  KNPFAPTLHF  NYRYFETDAP  KGAPGAPRQW
barley          GVCVHSRVLQ  DGNVFEKAGV  NVSAVIGVCP  RSAYRAAKG.  AAKNGAA..D  GHKAGPVPFF  SAGISSVLHP  KNPFAPTLHF  NYRYFETDAP  KDVPGAPRSW
soybean         GGGGISRVLQ  DGAVWEKAGV  NVSVVYGVMP  PDAYRAAKGV  PT.......D  Q.KPGPVPFF  AAGISSVLHP  KNPFAPTLHF  NYRYFETDAP  KDAPGAPRQW
tobacco         GGGGHSSVLQ  DGAVFEKAGV  NVSVVYGVMP  PEAYRAARPT  DN.......G  NVKPGPIPFF  AAGVSSVLHP  KNPFAPTLHF  NYRYFETDAP  KDAPGAPRQW
consensus       G....SRVLQ  DG.V.EKAGV  NVS.V.GV.P  ..AYR.....  ..........  ..K.GP.PFF  .AG..SVLHP  KNPFAPTLHF  NYRYFETDAP  K..PGAPR.W

                301                                                351                                                400
maize CPX1      WFGGGTDLTP  SYIIEEDVKH  FHSVQKQACD  KFDPSFHPRF  KKWCDDYFYI  KHRNERRGLG  GIFFDDLNDY  DQEMLLNFAT  ECADSVLPAY  IPIIERRKNT
maize CPX2      WFGGGTDLTP  SYIIEEDVKH  FHSVQKQACD  KFDPSFHPRF  KKWCDDYFYI  KHRNERRGLG  GIFFDDLNDY  DQEMLLNFAT  ~~~~~~~~~~  ~~~~~~~~~~
barley          WFGGGTDLTP  SYLIEEDVKH  FHSVQKQTCD  KFDPSFYPRF  KKWCDDYFYI  KHRNERRGLG  GIFFDDLNDY  DQDMLLNFAT  ECAGSVIPAY  IPIIERRKDT
soybean         WFGGGTDLTP  AYIFEEDVKH  FHSIQKQACD  KFEPTFYPRF  KKWCDDYFYI  KHRGERRGLG  GIFFDDLNDY  DQEMLLSFAT  ECANSVIPAY  LPIIEKRKDL
tobacco         WFGGGTDFTP  AYIFEEDVKH  FHSVQKAACD  KFDASFYPRF  KKWCVDYFYI  KHRDERRGLG  GIFFDDFNDY  DQEMLLSFST  ECANSVIPAY  IPIVEKRKDT
consensus       WFGGGTDLTP  .Y..EEDVKH  FHS.QK..CD  KF...F.PRF  KKWC.DYFYI  KHR.ERRGLG  GIFFDD.NDY  DQDMLL.F.T  ECA.SV.PAY  .PI.E.RK..

                401                                                451                 477
maize CPX1      PFNEEHRAWQ  QLRRGRYVEF  NLVYDRGTTF  GLKTGGRIES  ILVSLPLTAR  WQYDHKPEEG  TEEWKLLEAC  INPKDWI
maize CPX2      ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~~~
barley          PFNEEQKAWQ  QVRRGRYVEF  NLVYDRGTTF  GLKTGGRIES  ILVSLPLTAR  WEYDHKPEEG  SEEWKLLDAC  INPKEWL
soybean         PFNDHQKAWQ  QLRRGRYVEF  NLVYDRGTTF  GLKTGGRIES  ILVSLPLTAR  WEYDHKPEEG  SEEWKLLDAC  INPKEWI
tobacco         PFTDKHKAWQ  QLRRGRYVEF  NLVYDRGTTF  GLKTGGRIES  ILVSLPLTAR  WEYDHKPEEG  TEEWKLLDAC  INPKEWI
consensus       PF.....AWQ  Q.RRGRYVEF  NLVYDRGTTF  GLKTGGRIES  ILVSLPLTAR  W.YDHKPEEG  .EEWKLL..C  INPK.W.
```

Figure 2.4 Amino acid sequence alignment of plant coproporphyrinogen III oxidase proteins. The undetermined sequence of maize CPX2 is represented by the symbol ~.

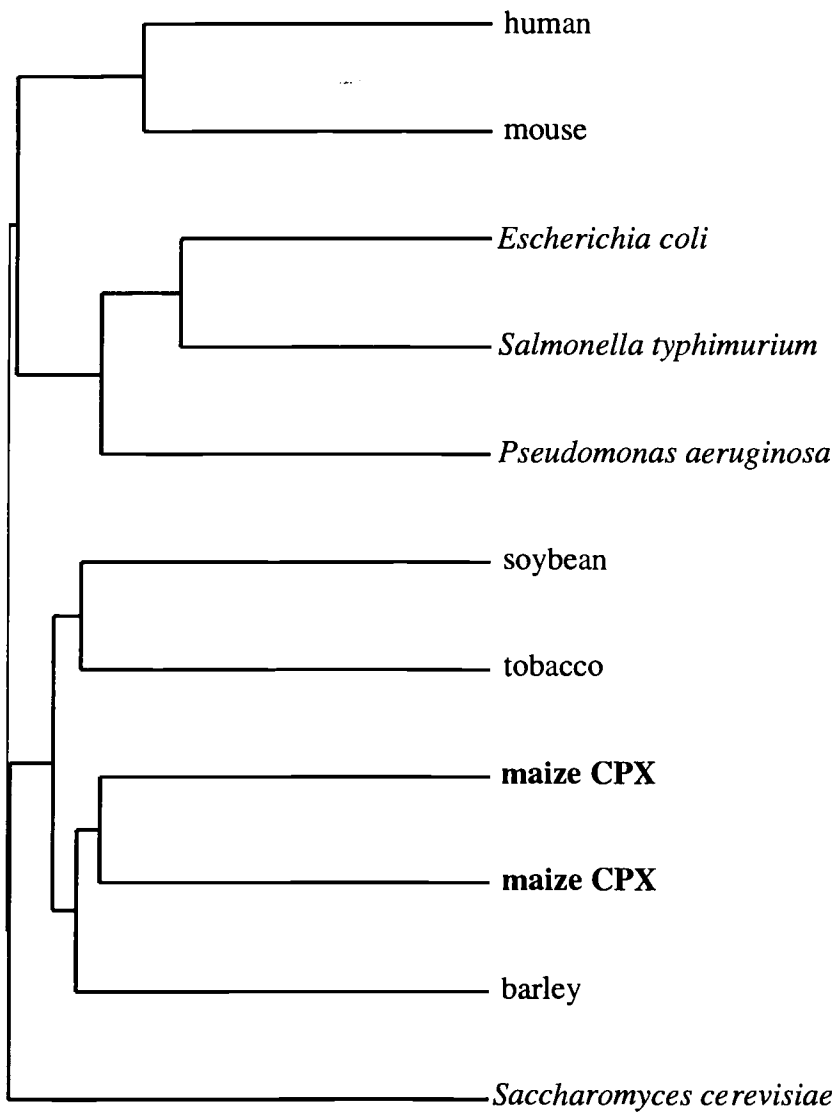

Figure 2.5 The amino acid homology of coprogen was compared between several organisms. Only the amino-terminal portion of coprogen, the equivalent of exon 1 in barley, was used in the alignment.

sequence (von Heijne, Steppuhn, and Herrmann 1989) and recognizes the potential protease cleavage sites (Gavel and von Heijne 1990). ChloroP, a more recent program with higher accuracy than PSORT for prediction of chloroplast transit sequences, was used to analyze the amino-terminal extensions of the maize CPX products. This program provides a more rigorous detection of conserved cleavage motifs using the consensus VR↓AAAVxx, based on a motif-finding algorithm (Bailey and Elkan 1994).

In order to develop a perspective of ChloroP and its predictions relative to enzymes with putative and documented localizations, I analyzed the amino-terminal regions of other plant coprogen enzymes and that of maize protoporphyrinogen IX oxidase (PPO), the following enzyme of tetrapyrrole biosynthesis (table 2.1). Each putative or verified chloroplast-localized enzyme scored a prediction value between 0.55 and 0.57; all are considered positive predictions of chloroplast localization. The mitochondrial-localized product PPO-2 scored a prediction value of 0.44; this is a negative prediction that falls below the 0.5 cut-off of a predicted chloroplast transit peptide.

CPX1 of maize was predicted by ChloroP to be a chloroplast transit peptide (score 0.58), consistent with that of all other plant coprogen enzymes studied. By homology to the putative peptide cleavage site of barley, a similar cleavage site could be predicted between amino acid residues 59 and 60 of CPX1. However, the ChloroP program predicted a better fit with the consensus for a processing-peptidase cleavage site preceding this region, between amino acid residues 56 and 57 (figure 2.6). Thus, the predicted mature CPX1 enzyme has 343 amino acid residues and a molecular weight of 38.6 kDa.

Table 2.1 Localization predictions for coproporphyrinogen III oxidase (CPX, CPO, and Coprogen) and protoporphyrinogen IX oxidase (PPO) enzymes. N/S refers to sequences not yet submitted to GenBank.

protein/organism	GenBank #	localization	source	ChloroP prediction
CPX-1 maize	N/S	putative: chloroplast stroma	this study	✓ chloroplast 0.582038
CPX-2 maize	N/S	putative: mitochondrial	this study	× chloroplast 0.477216
CPO barley	X82830	putative: chloroplast stroma	Kruse, Mock, and Grimm 1995	✓ chloroplast 0.567232
CPO tobacco	X82831	chloroplast stroma (pea import assay)	Kruse, Mock, and Grimm 1995	✓ chloroplast 0.559707
Coprogen soybean	X71083	putative: chloroplast stroma	Madsen *et al.* 1993	✓ chloroplast 0.570909
PPO-1 tobacco	Y13465	chloroplast stroma and thylakoid (pea import assay, immunology)	Lermontova *et al.* 1997	✓ chloroplast 0.578826
PPO-2 tobacco	Y13466	mitochondrial (pea import assay, immunology)	Lermontova *et al.* 1997	× chloroplast 0.446655

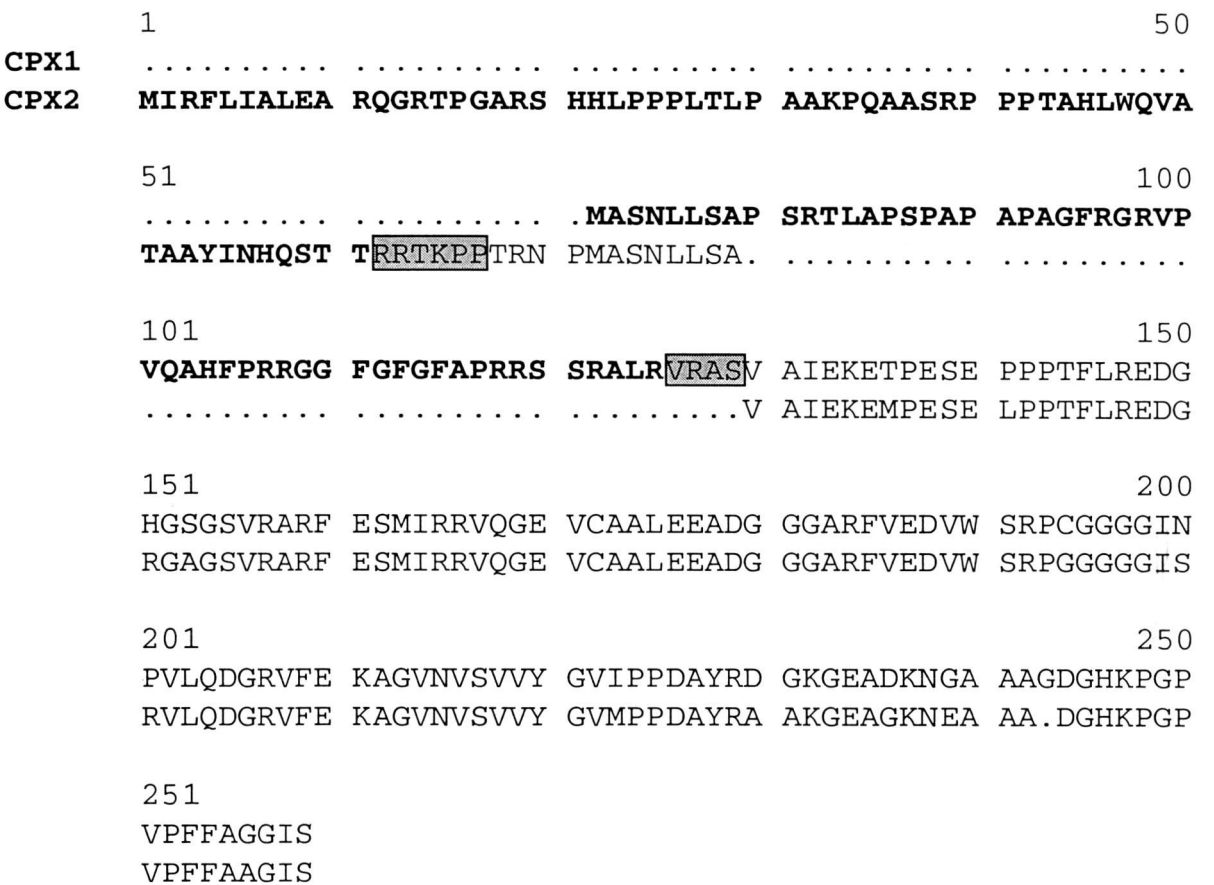

Figure 2.6 Amino acid alignment of the amino-terminal regions of CPX1 and CPX2. The putative targeting peptides are in bold and the predicted peptide cleavage sites are in shaded boxes.

CPX2 also has a potential amino-terminal extension; however, as a result of the *Cpx2* deletion in relation to *Cpx1* and the upstream in-frame methionine codon, the extension is not homologous to that of CPX1. In fact, the predicted chloroplast transit peptide is almost entirely deleted in the CPX2 protein, including the cleavage sequence. When analyzed with the ChloroP program, the amino-terminal extension of CPX2 is predicted not to be a chloroplast transit peptide (score 0.48). CPX2 was analyzed with PSORT and was predicted to be a mitochondrial matrix protein (score 0.57) based on the following parameters: the transit peptide amino acid composition, the potential for an amino-terminal amphiphilic α-helix, the presence of a hydrophobic domain following this region, and the recognition of a peptidase cleavage site (vonHeijne, Steppuhn, Herrmann 1989; Gavel and von Heijne 1990). The mitochondrial-localization prediction is based on data generated from both plant and animal mitochondrial targeting peptides. A recent analysis comparing these peptides has shown that there is little difference in the nature of mitochondrial-targeting peptides between these organisms (Schneider *et al.* 1998). The predicted transit peptide junction motif is RRT↓KPP (amino acids 62-67) (figure 2.6). This motif is loosely conserved with the consensus RXY↓S/A (Gavel and von Heijne 1990) with an arginine residue at position –2, but does not have a serine or alanine residue at position +1. The predicted localization of the mature CPX2 enzyme is, thus, mitochondrial, with a processed size of 419 amino acids and a molecular weight of 39.9 kDa.

2.5 Discussion

We have determined by homology that the maize genome encodes two *Cpx* genes. This is the first finding of multiple copies of a coprogen-encoding gene in a higher plant. These genes were mapped to syntenous regions of the genome, consistent with the occurrence of a duplication event after the speciation of maize.

In accordance with the prediction that the *Cpx* genes are duplicates, the CPX proteins of maize are closely related to one another. The evolutionary relationships of several kingdoms are reflected by the divergence of coprogen enzymes; maize CPX enzymes are closely conserved to those of other plants, and more distantly to coprogen of mammals, yeast, and bacteria. In particular, the maize CPX products both share the three highly invariant regions characteristic of coprogen enzymes, which indicates that each product may be catalytically active. They have homology with soybean coprogen, which has been shown to complement a yeast coprogen deficiency (Madsen *et al.* 1993). It is likely that both maize *Cpx* genes encode a functional coprogen enzyme.

The *Cpx* genes of maize significantly differ from one another in the preserved 5' coding region as the result of a 150 bp deletion in *Cpx2* as compared to *Cpx1*. The deletion results in the loss of 50 amino acids that are predicted to direct chloroplast translocation. However, the open-reading frame of *Cpx2* may begin further upstream than the *Cpx1* gene, extending it another 213 bases. The differences between the *Cpx1* and *Cpx2* genes appear to have resulted from a series of small deletion events which created an extended open-reading frame contiguous with exon 1. Computer analysis of the translated product of this 5' extension predicts a transit peptide directing the protein to the mitochondrial matrix.

Cpx1 is more highly conserved to the coprogen-encoding genes of barley, tobacco, and soybean than is *Cpx2*; therefore, it is likely to be more similar to an ancestral plant coprogen gene. After duplication, the *Cpx2* progenitor was altered through a series of deletion events, resulting in the acquisition of a new open-reading frame within a region with homology to the 5' untranslated region of *Cpx1*. Thus, I hypothesize that CPX1 is chloroplast-localized like other plant coprogens and CPX2 is mitochondrion-localized, as the result of a series of small deletion events during the divergence of *Cpx2*.

In several studies, plant coprogen enzymes have been determined or predicted to be chloroplast-localized proteins (Kruse, Mock, and Grimm 1995a; Heijne *et al.* 1989). There is only one report describing coprogen activity in a mitochondrial fraction from tobacco *(Nicotiana tabacum)* (Hsu and Miller 1970), but this observation was later criticized because the mitochondrial fraction contained broken chloroplasts (Smith, Marsh, and Elder 1993). Coprogen activity was determined to be present in the chloroplast fraction of etiolated pea leaves, and not in isolated mitochondria (Smith, Marsh, and Elder 1993). The coprogen enzymes of soybean, tobacco, and barley have putative chloroplast transit peptides, and in *in vitro* translation experiments both soybean and tobacco coprogen proteins were imported into isolated pea chloroplasts (Kruse, Mock, and Grimm 1995a; Madsen *et al.* 1993). Therefore, the possibility that CPX1 is located in the chloroplast is consistent with previous findings of CPX in plants and with the fact that the complete tetrapyrrole biosynthetic pathway occurs within the chloroplast (Reinbothe and Reinbothe 1996).

I have presented data that supports the interpretation that CPX2 does not have a chloroplast transit peptide and, thus, is less likely to be chloroplast-localized. Moreover, the amino-terminal extension of CPX2 appears to be a mitochondrial-targeting peptide. Therefore, I hypothesize that CPX2 is a mitochondrial-localized protein. Despite the fact that this is not consistent with coprogen in other plants, mitochondrial coprogen activity is a logical prediction because the activities of the subsequent two enzymes in heme biosynthesis, protoporphyrinogen IX oxidase and ferrochetalase, were associated with plant mitochondria (Jacobs and Jacobs 1995). This prediction also parallels a recent study of protoporphyrinogen IX oxidase in tobacco; two different cDNA sequences of this gene were isolated and identified as plastid and mitochondrial isoforms (Lermontova *et al.* 1997). It is believed that the product of coprogen, protoporphyrinogen IX, is translocated from the chloroplast to the mitochondria, providing a precursor for mitochondrial heme biosynthesis (Smith, Marsh and Elder 1993). A maize mitochondrial coprogen would support the modification of an earlier precursor of heme, coproporphyrinogen III. Thus, the presence of a mitochondrial coprogen enzyme suggests that an earlier precursor is transferred from the chloroplast to the mitochondria.

The discovery of distinct and independent locations of each CPX enzyme might be correlated with individual roles within the cell. A chloroplast localization of CPX1 would be consistent with an enzyme involved in tetrapyrrole biosynthesis in this location. Likewise, the role of a mitochondrial CPX2 enzyme would be consistent with a primary function in mitochondrial heme biosynthesis. Because plant cells have varying needs for both heme and chlorophyll, the production of each tetrapyrrole could be compartmentalized in maize and its biosynthesis regulated independently. Differences in

primary function and regulation should correlate with difference in expression patterns and mutant phenotypes, as well as with differences in localization. These topics are addressed in chapters 3, 4, and 5, respectively.

2.6 Acknowledgements

I would like to thank John Fowler for providing the lambda-MAX cDNA library. I would also like to thank Alice Barkan for providing the lambda-ZAP cDNA library and her advice concerning chloroplast transit peptides and the program ChloroP. Finally, I would like to thank Pioneer HiBred International, Inc. for providing the lambda-DASH II genomic library.

3. Analysis of the Relative Transcriptional Expression of the Maize *Cpx* Genes

3.1 Abstract

The activity of coproporphyrinogen III oxidase (coprogen) is required for the biosynthesis of tetrapyrroles. In maize, we have determined that there are two coprogen-encoding genes (*Cpx*), both of which appear to contain conserved open-reading frames that could translate into functional enzymes. In this study, I have detected both *Cpx* transcripts in all the tissue types tested of inbred line W-22. The relative levels of the transcripts vary with tissue type. In seedling shoots, the *Cpx1* transcript is detected at levels roughly 3-4 fold higher in relation to the *Cpx2* transcript. In seedling roots, both transcripts are detected at similar levels. The upregulation of *Cpx1* transcription may be correlated with a role in chlorophyll biosynthesis in shoot tissue; however, there is no indication of light-regulated *Cpx* transcription. The lower levels of *Cpx1* transcripts in root tissue and of *Cpx2* transcripts in both shoots and roots may correspond to a role in general tetrapyrrole biosynthesis, especially in the maintenance of cellular heme levels. The differences in the expression of *Cpx1* in relation to that of *Cpx2* is consistent with a divergence in their roles within the cell.

3.2 Introduction

Heme and chlorophyll are both products of the tetrapyrrole biosynthetic pathway, but their relative synthesis is regulated. Because heme is required for respiration and living cells respire, heme must be produced by cells throughout development. When

dark-grown barley seedlings are exposed to 5 hours of illumination and a heme and chlorophyll precursor, C^{14}-aminolevulininc acid, is administered during this time, the specific activity of purified protoheme and pheophorbide (chlorophyll derivative) are nearly identical (Castelfranco and Jones 1975). New protoheme is formed but the heme pool does not accumulate; therefore, heme must be degraded at a rate similar to its synthesis. The half-life of exogenously applied heme taken up by etiolated barley shoots was estimated to be 8-9 hours (Hendry and Stobart 1978). Genes involved in the biosynthetic pathway of tetrapyrroles, then, must be expressed constitutively in order to continuously provide heme. In contrast, chlorophyll production is specific to certain tissues and at certain stages of development and, therefore, its production must be regulated to meet the demands of the cell.

Because the biosynthesis of both heme and chlorophyll are common up to the intermediate protoporphyrinogen IX, the regulation of their production must occur after the branch point in biosynthesis. In fact, the regulation of tetrapyrrole biosynthesis is much more complex than that. In chapter 1, I summarized the key regulatory points, but there are likely to be many more subtle ones that moderate the flow of tetrapyrrolic intermediates, preventing the build-up of these phototoxic compounds. The level of tetrapyrroles within the cell depends on both biosynthesis and degradation. Through a balance between these processes, cellular heme levels are maintained and chlorophyll levels vary dramatically (Castelfranco and Jones 1975).

Transcriptional control is one of the mechanisms by which tetrapyrrole biosynthesis is regulated. Etiolated angiosperm seedlings contain all the enzymes necessary to produce tetrapyrroles (Nadler and Granick 1970). The light-induced

production of chlorophyll is, in part, made possible by the upregulation of biosynthetic enzymes. There appears to be two categories of transcriptional control associated with chlorophyll production. The first category is transcriptional upregulation in response to light. For example, the level of δ-aminolevulinic acid dehydratase transcripts in etiolated pea seedlings increases 20-fold after exposure to light (Witty *et al.* 1993). The second category is a tissue-specific transcript level that affects the capacity of that tissue to produce chlorophyll, implying developmental regulation. An example of this is the transcript level of the plastidal isoform of tobacco protoporphyrinogen IX oxidase (Lermontova *et al.* 1997). This transcript was shown to be present in leaf tissue, at higher levels in younger leaves than older ones, and it was not detected in root tissue. A similar observation was made with respect to the level of coprogen transcripts in barley seedlings (Kruse, Mock and Grimm 1995a). The level of coprogen transcripts was considerably higher in leaves and coleoptiles than in roots, and it was higher in younger than in older leaf tissue in both barley seedlings and tobacco plants. Both light-regulated and developmentally-regulated transcription of tetrapyrrole biosynthetic enzymes are mechanisms that participate in the regulation of chlorophyll biosynthesis.

In chapter 2, I hypothesized that both *Cpx* genes of maize encode functional coprogen enzymes because the putative catalytic region of each product is highly conserved to that of coprogen in other organisms. In order to investigate the possibility that both genes are expressed, I used RT-PCR analysis to detect trancriptional products from each *cpx* gene and to compare their relative expression levels.

3.3 Methods

Total RNA was extracted from the stem, flag leaf, silks, tassels, and immature ears of adult W-22 plants according to Wessler (Wessler 1994). RNA was also extracted from the shoots and roots of 10-11 day-old seedlings of W-22 that were either grown in 12h light/12 h dark conditions or 24 h dark conditions at 25-29°C. 19-DAP embryo and kernel RNA from W-22 were obtained from Carol Rivin.

cDNA was produced from total RNA at 50°C using the SuperScript Preamplification System (Life Technologies) and a poly-T primer. RT-PCR amplification was performed according to the following procedure using the cDNA product as template: 25µl reactions were run in 0.2mM dNTP mix, 10% sucrose, 10% DMSO, 1.5mM $MgCl_2$ with 0.8 U Taq polymerase (Qiagen). Primers specific to both maize *Cpx* genes (see figure 3.1) and to that of maize actin (Pioneer HiBred Intl.) were generated for the analysis. Primers B (5'-CCGCCGAACCACCATTGTCTTGGT-3'), C (5'-ACCCAA ACCCAATGGCGTCCAACCTC-3'), ZmacT1 (5'-CACTGGAATGGTCAAGGCCGGT TTC-3'), and ZmacT2 (5'-AACCGTGTGGCTCACACCATCACCT-3') were synthesized by the Central Services Laboratory (CSL) at Oregon State University. For semi-quantitative analysis, cDNA templates were serially diluted four-fold into sterile dH_2O, stored at -20°C, and used within one month of preparation. Amplification was conducted in a TwinBlock Easy Cycler (Ericomp) or a DeltaCycler I Easy Cycler (Ericomp) using 5 min at 94° (once), 1 min each at 94° and 62°, 2 min at 72° (35 times), and 5 min at 72° (once). Both *Cpx-1* and *Cpx-2* cDNAs were amplified with primer set C-B and the actin control was amplified with primers set ZmacT1-ZmacT2. RT-PCR products were separated on ethidium bromide stained 1.2% agarose gels, and the gels

were destained for a minimum of 30 minutes before visualization and quantification using the FMBioII fluorimager and software (Hitachi). Each quantitation value was normalized for the amount of actin amplification in that reaction.

3.4 Results

3.4.1 The Transcriptional Expression of *Cpx*

Transcripts from the *Cpx1* and *Cpx2* genes were detected using RT-PCR and a single primer set that amplifies a different-sized product from each *Cpx* transcript (figure 3.1). Various tissues from inbred line W-22 were tested for the expression of the *Cpx* genes using the RT-PCR assay. The different tissues were selected in order to provide at least one example of *Cpx* expression in embryos, seedlings, adult plants, and reproductive, somatic, photosynthetic, and non-photosynthetic tissues. In all the samples, the transcripts of both *Cpx* genes were detected (figure 3.2A).

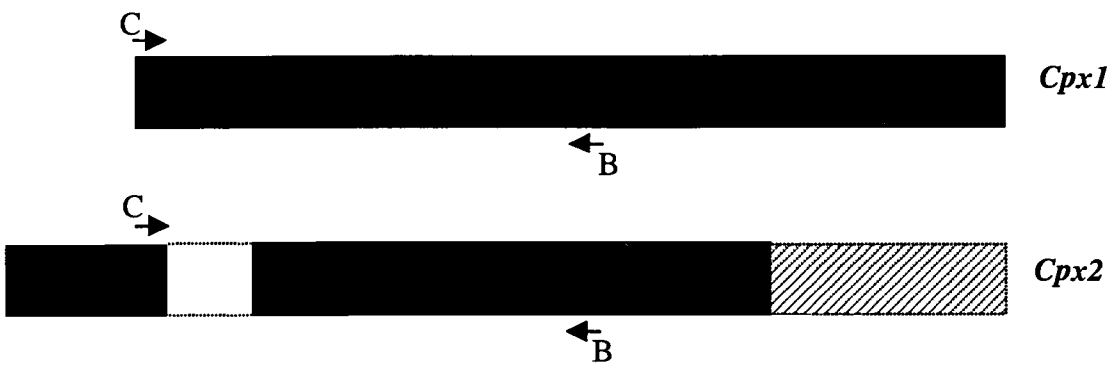

Figure 3.1 The RT-PCR amplification scheme for *Cpx*. Both *Cpx 1* and *Cpx2* are amplified by a single primer set (primers C to B), producing different-sized products. The arrows represent the primer annealing sites, the grey boxes are the individual *Cpx* transcripts, the unshaded box is the region deleted in *Cpx2*, and the hatched box is the unsequenced region of *Cpx-2*.

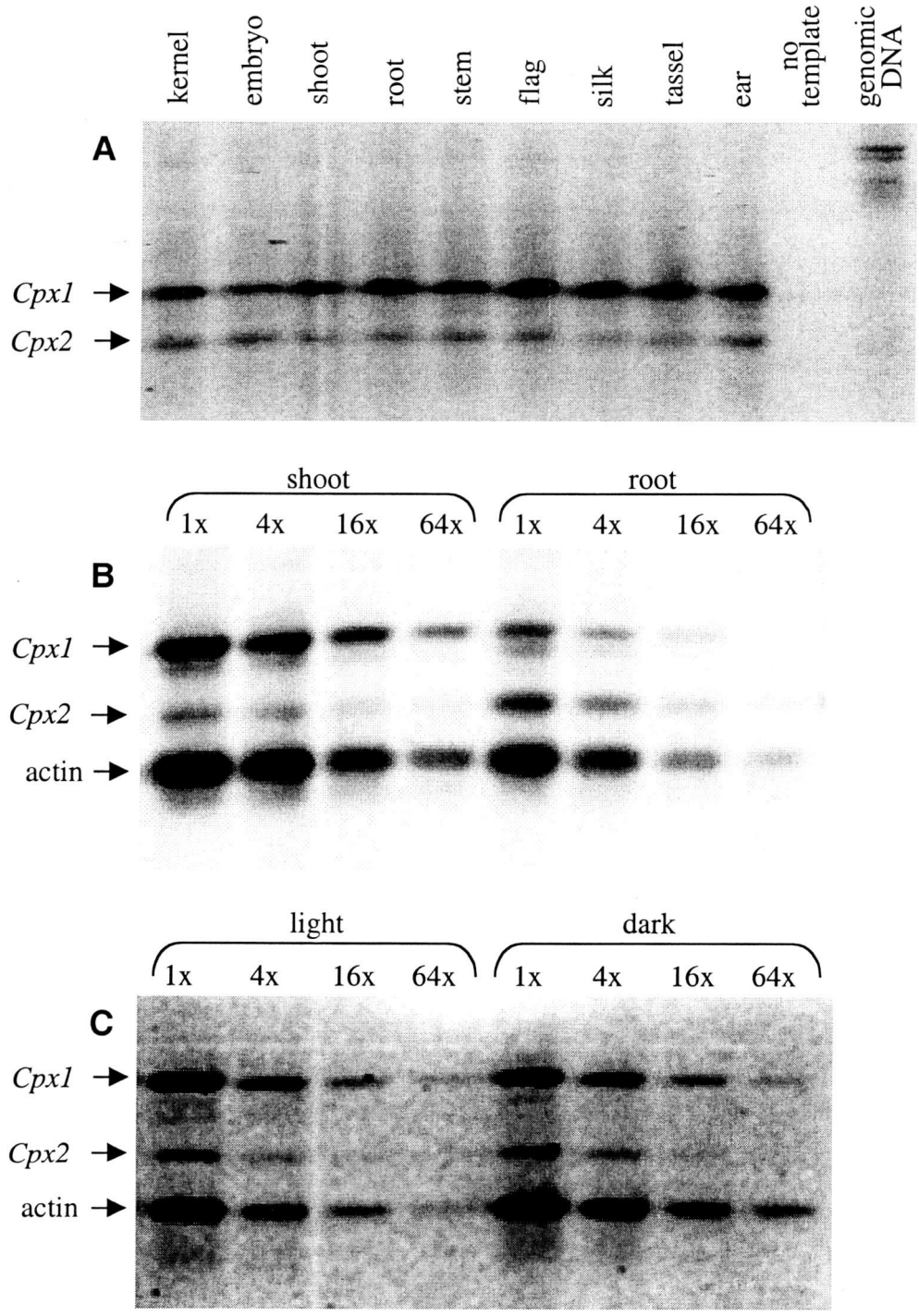

Figure 3.2 RT-PCR analysis of W-22; the amplification products were separated by agarose gel electrophoresis and visualized with ethidium bromide staining. The reverse images are shown. The *Cpx* cDNAs were amplified from various tissue samples (A). An RT-PCR template dilution series was amplified from seedling shoot and root tissue (B) and from seedling shoots grown in the presence or absence of light (C). The template dilution factor is indicated above.

3.4.2 The Relative Steady-State Levels of *Cpx* Transcripts

The comparison of tissues above suggested that *Cpx1* and *Cpx2* were differentially expressed in some tissues, but not others. A more quantitative comparison was made using cDNA generated from seedling shoot and root tissue (figure 3.2B) using a serial dilution RT-PCR approach (Nebenführ and Lomax 1998). Four-fold serial dilutions were made from each cDNA preparation and all the dilution samples were PCR-amplified. As template concentration becomes limiting, the amount of amplified product should be proportional to the template concentration, although the amplification efficiency of each product may vary. As an internal control of RNA level and quality, a primer set specific to the maize actin cDNA was added to each sample. Amplification of the actin cDNAs did not interfere with *Cpx* amplification.

The relative amounts of *Cpx* products in different tissues were normalized to the actin control (figure 3.3). The relative amounts of *Cpx1* and *Cpx2* products are quite different in shoots and roots. In shoot tissue, the level of the *Cpx1* amplification product is roughly 3-fold higher than the *Cpx2* product while in root tissue the relative amount of amplified products are very similar. No significant difference was detected in the levels of *Cpx-2* in either tissue.

3.4.3 The Effect of Light on *Cpx* Transcript Levels

In a similar experiment, the *Cpx* amplification products were compared from light- and dark-grown seedling shoots (figure 3.2C). The relative levels of the *Cpx1* and *Cpx2* products, normalized for the amount of actin amplification, were very similar (data

not shown). Light-grown tissue appears to have the same content of *Cpx* transcripts as tissue grown in the absence of light.

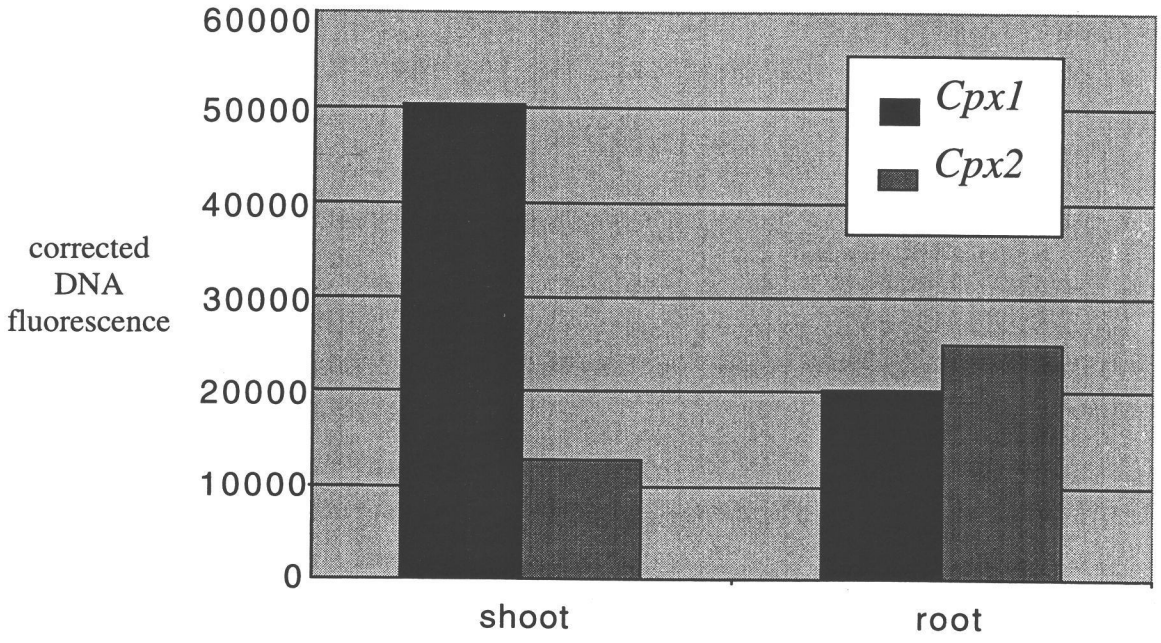

Figure 3.3 RT-PCR amplification levels. The level of actin amplification in each sample was used to normalize the relative amplification levels of *Cpx1* and *Cpx2* products from seedling shoot and root samples. The experiment was repeated a total of five times and a typical experiment is shown here.

3.5 Discussion

Both *Cpx1* and *Cpx2* transcriptional products were detected in all the tissues tested, consistent with the prediction that each gene encodes a functional protein. Assuming this prediction is valid, both enzymes appear to be expressed throughout the

plant. This data suggests that both genes play a role in tetrapyrrole production in all tissues, but it does not define their relative functions.

The amount of amplified product from *Cpx1* and *Cpx2* is proportional to the amount of template when template concentration is the limiting factor, but the efficiency of amplification of each product may vary. Therefore, although the RT-PCR assay is an indicator of the relative transcript levels of each *Cpx* gene, it is a better measurement for comparisons of the same amplification product in different tissues between *Cpx1* and *Cpx2* because some template bias may occur. The primers are identical to the sequence of the *Cpx1* gene and each has two base differences to that of *Cpx2*. However, these base differences are located near the 5' end of the primer and should have very little effect on the relative amplification of each gene product. In a semi-quantitative assay, *Cpx1* transcript levels are 2.9 ± 0.3 fold higher in seedling shoots than in roots in relation to the actin control, perhaps reflecting the higher CPX demand for chlorophyll biosynthesis in photosynthetic tissue. In comparison, *Cpx2* transcript levels do not appear to vary significantly in relation to the actin control in shoots and roots. The observation of a base level of transcription of *Cpx2* is consistent with a role in constitutive tetrapyrrole production.

There does not appear to be an affect from light exposure on the transcription of either *Cpx* gene. This analysis only measured the relative levels of *Cpx* transcripts from shoot tissue as a whole. Kruse, Mock, and Grimm (1995a) didn't see a light-dependent expression of barley and tobacco coprogen, but detected a higher level of coprogen transcripts in young leaf tissue compared to that in older leaf tissue in both barley seedlings and tobacco plants. There may be more localized expression patterns of *Cpx* in

maize that are not detected by this experiment. Both *Cpx* genes are expressed constitutively and the relative level of expression is not light-regulated.

The expression pattern of the *Cpx* genes is consistent with tissue-specific regulation of the transcriptional level of *Cpx1* and general low-level of *Cpx2* transcription in all tissues. I hypothesize that this reflects a somewhat different role for the CPX enzymes. *Cpx1*, by sequence analysis, appears to be targeted to the chloroplast where it plays a critical role in chlorophyll synthesis, as well as heme synthesis. *Cpx2* appears to be mitochondrial-localized and, therefore, limited to heme synthesis. The invariant expression of *Cpx2* is consistent with this role, while *Cpx1* expression is expected to increase in photosynthetic tissue. Further tests of this theory using mutant analysis are presented in chapter 4.

3.6 Acknowledgements

Thanks to Andreas Madlung for his counsel regarding semi-quantitative RT-PCR.

4.0 Identification and Analysis of *Cpx* Mutant Alleles

4.1 Abstract

The analysis of systems disrupted for the expression individual of genes of a gene family can be useful in assigning functions to specific gene products. In our lab, a mutant allele of *Cpx1* was identified that has a Mutator transposable insertion within the open-reading frame *(cpx1-1)*. Using the trait utility system for corn (TUSC), in collaboration with Pioneer HiBred International, we were able to identify five more Mutator transposon insertion mutants within the maize *Cpx* genes; one within *Cpx1*, *cpx1-116*, and four within *Cpx2*, *cpx2-104*, *cpx2-323*, *cpx2-578*, and *cpx2-105*. Through genomic PCR analysis, the transposable element insertion site of each mutant was assigned and the region sequenced. One mutant family for each gene was characterized for the presence of a transcript from the mutated gene using RT-PCR analysis. The results of the experiments indicate that *cpx1-1* and *cpx2-578* produce a mutant transcript from their *Cpx* mutant alleles. Translational products produced from the *cpx1-1* transcript would not have an N-terminal transit peptide. The *cpx2-578* transcript appears to contain the entire open-reading frame, but would produce a truncated CPX2 product. Plants heterozygous for each allele appear wild-type in phenotype. *cpx1-1* homozygotes, as previously described, have a defective kernel phenotype that is subject to a background effect. In inbred line H-99, plants homozygous for *cpx1-1* develop into yellow seedlings before becoming necrotic. From my analysis, I predict that the mutant *cpx1-1* transcript is unable to provide CPX1 activity within the chloroplast; therefore, it cannot participate in heme and chlorophyll biosynthesis in that location. The deficiency of chlorophyll may be the cause

of the yellow seedling, lethal phenotype. The *cpx2-578* homozygotes do not appear to have a mutant phenotype, presumably because the *Cpx-1* gene product is able to complement the CPX2 deficiency or because a CPX2 deficiency is not a serious biochemical problem under normal growth conditions.

4.2 Introduction

The tetrapyrrole biosynthetic pathway has been studied for over a century, primarily because of the effect of abnormal porphyrin production in humans. Many syndromes caused by hereditary porphyrias have been described as the result of mutations in specific heme biosynthetic enzymes (Dailey 1990). One characteristic set of abnormalities associated with the accumulation of porphyrin intermediates is acute solar photosensitivity and an unusually high urine content of porphryins. Hereditary coproporphyria is inherited as a dominant autosomal trait that manifests as a deficiency in coproporphyrinogen III oxidase (coprogen). People who have this disfunction develop skin lesions after exposure to light and experience bouts of acute porphyria, although in some cases the condition remains latent throughout life. The general symptoms of acute porphyria are the following: acute abdominal pain with vomiting and constipation, psychiatric features such as anxiety, depression, disorientation, confusion, and delirium, and neurological disorders such as peripheral neuropathy, respiratory embarrassment, and grand mal seizures.

Mutants in this pathway were created in tobacco by transformation with a coproporphyrinogen III oxidase (*Cpo*) antisense cDNA expression construct (Kruse, Mock and Grimm 1995b). The transformed plants in this study had differing levels of

Cpo transcription, reduced coprogen activity, and coproporphyrinogen III accumulation. The resulting phenotypes ranged from symptomless plants to plants with severe photodynamic damage, the severity correlated with a decrease in the level of *Cpo* transcripts.

The build-up of high levels of porphyrin intermediates leads to the generation of reactive oxidative species (Smith 1987). The tobacco *Cpo* mutants were characterized for evidence of oxidative stress and shown to have increased levels of superoxide dismutase and decreased levels of tocopherol. Both results are correlated with an increase in cellular oxidative defense mechanisms. In a more recent publication, the oxidative stress response of these plants, and those transformed with an antisense uroporphyrinogen decarboxylase construct, is characterized as having a more global intracellular effect (Mock *et al.* 1998). It is believed that the accumulated porphyrins leak out from the plastid and affect other cellular compartments, stimulating various cellular oxidative defense systems.

Recently, a uroporphyrinogen decarboxylase mutant of maize, *Les22*, was described as a lesion mimic (Hu *et al.* 1998). This mutation is dominant and the heterozygotes have a phenotype of scattered tiny necrotic lesions that begin to develop on the primary leaf of three to four week-old plants. The lesions are light-dependent and remain tiny, but increase in number to eventually cover the entire leaf blade. *Les22* homozygotes develop into yellow seedlings before dying. The level of detectable uroporphyrin III accumulation in heterozygotes is approximately 3-fold above that of wild-type and the level increases up to 60-fold in homozygotes. Remarkably, plants that have deregulated tetrapyrrole biosynthesis develop a photosensitivity similar to that of humans, this phenotype has been termed phytoporphyria.

In our lab, a defective kernel mutant of maize was determined to have an insertion within the coprogen-encoding gene, *Cpx1* (Hardeman and Strom, unpublished data). There is no phenotype associated with heterozygotes, but the phenotype of homozygotes for this mutation, *cpx1-1*, are defective kernels that germinate, produce a primary root that is root-hairless, and do not develop a histologically-identifiable shoot (Sollinger 1994). When crossed into the inbred line H-99, the *cpx1-1* defective kernels do develop into yellow seedlings before dying. In chapter 2, I described a second coprogen-encoding gene of maize: *Cpx-2*. In collaboration with Pioneer HiBred International, Inc., we identified new mutants in each *Cpx* gene. The following study is the identification of plants carrying heritable mutant alleles, an analysis of each mutant allele, and a preliminary characterization of the expression of these mutant alleles and phenotypes associated with them.

4.3 Methods

4.3.1 Identification of Mutator Insertional Mutants

Total genomic DNA was isolated following the microprep method (Freeling and Walbot 1994) from leaf tissue of plants growing at the OSU Botany Farm. The lines were obtained from Pioneer as families putatively segregating for Mutator insertions within either of the *Cpx* genes. The alleles were considered heritable if they could be identified in these families, which are the progeny of the individual Mutator plants tested by Pioneer. For the initial screen, approximately equivalent quantities of DNA were pooled into groups of 4-10 and screened through PCR amplification using Mu-B and Mu-C, separately. Primers C and B are listed in chapter 3. Primer Mu (5'-

AGAGAAGCCAACGCCA(AT)CGCCTC (CT)ATTTCG TC-3') is a degenerate primer that can specifically amplify the inverted repeats of several Mutator transposable elements. The PCR amplification conditions were the same as in the RT-PCR analysis of chapter 3. 10 µl of a 12.5µl reaction was loaded onto a 1.0% agarose gel in Tris-Borate buffer (Sambrook, Fritsh and Maniatis 1989) and separated by electrophoresis. The resultant gel was Southern blotted (Sambrook, Fritsh and Maniatis 1989) and hybridized at 65° as described in chapter 2 using the probe pCBS, a cloned C-B PCR product of *cpx-1* genomic DNA. The blots were washed as in chapter 3 and the radioactivity was detected using a phosphoimager detection screen (Molecular Dynamics) for a few hours or overnight. The results were analyzed using the ImageQuant program (Molecular Dynamics). Positive pools were re-screened as individuals following the same procedure. Individual PCR products were excised from the agarose gel, purified using the Geneclean II kit (Bio 101, Inc.), and sequenced at the Central Services Lab (CSL) using Mu as the sequencing primer.

Plants segregating for each of the mutant alleles were tested for the zygosity of the mutant allele through PCR amplification across the site of Mutator insertion. The primers employed are shown in figure 4.2. The *cpx2-323* allele was analyzed using the primer set UPST/GFP (5'-AGGGATCCATGATCCGTTTCCTTATC-3')-A (5'-CGGGCCAGGCTTGTGTCCATC-3') in order cross the upstream insertion site of this allele. The *cpx1-1* and *cpx2-578* alleles were analyzed using the primer set C-A. The *cpx1-116* and *cpx2-105* alleles were analyzed using the primer set P1 (5'-GGCGGCGGCATCAGCCG-3')-B and the amplification products were digested with the restriction enzyme BglII in order to specifically cleave the *cpx-2* products and

separate them from the *cpx-1* products. The PCR amplification conditions and product detection were the same as listed in chapter 3, except the thermocycler conditions were as follows: 1 min at 94° (one time); 1 min at 94°, 1 min at 54°, 2 min at 72° (5 times); 1 min at 94°, one min at 62°, two min at 72° (30 times); and 5 min at 72° (one time) in order to accommodate the lower annealing temperature of the primer UPST/GFP.

4.3.2 RT-PCR Analysis

RNA was extracted from the leaf tissue of maturing plants growing at the OSU Botany Farm and RT-PCR analysis was performed according to the methods in chapter 3. One individual was selected from each family that was carrying one of the Mutator insertion alleles *cpx1-1*, *cpx1-116*, *cpx2-104*, *cpx2-323*, *cpx2-578* and *cpx2-105*. The wild-type control used was the W-22 flag leaf sample in chapter 3. RNA was also isolated from the shoot tissue of two week-old seedlings homozygous for the *cpx1-1* (in inbred line H-99) or *cpx2-578* alleles and from W-22 seedlings as described in chapter 3. The *cpx1-1* and *cpx2-578* genomic DNA controls were prepared using the microprep method mentioned in the preceding paragraph. cDNA preparation and RT-PCR analysis was performed as in chapter 3 with the exception that all leaf RNA samples were treated with RNase-free DNase (Ambion) as per recommended by the SuperScript Preamplification System, removing all traces of genomic contamination. Also, the primer set UPST/GFP-Mu was amplified using the thermocycler conditions listed above. The primer sets used in individual or paired PCR sets were C-B, UPST/GFP-Mu, B-Mu, and ZmacT1-ZmacT2.The RT-PCR products were separated on a 1.2% agarose gel and the ethidium bromide-stained DNA was visualized using a fluorimager (Hitachi).

4.4 Results

4.4.1 Identification of *Cpx* Alleles and Mutator Insertion Points

The Trait Utility System for Corn (TUSC) was developed as a reverse genetics technique used for the identification of a Mutator transposible element insertion within a specific gene of interest (Meeley and Briggs 1995). Pioneer HiBred International, Inc. has automated this system to screen over 40,000 Mutator lines. In collaboration with Pioneer, these lines were screened for Mutator insertions within the *Cpx* genes. Two *Cpx*-specific primers, C and B, were used in conjunction with a Mutator-specific primer, Mu (figure 4.1). In independent experiments, primer sets C-Mu and B-Mu were used in the PCR screening of pooled genomic DNA from the Mutator lines. Of the nine candidate *Cpx* insertion mutants found in the screen, we were able to identify five heritable alleles of *Cpx;* one allele of *Cpx1* and four alleles of *Cpx2*. The Mutator insertion site of each allele was verified by sequencing amplification products produced by the PCR screen (table 4.1). The *cpx1-116* allele has an insertion within intron 1 of *Cpx1*. The *cpx2-104*, *cpx2-323*, *cpx2-578*, and *cpx2-105* alleles have Mutator insertions within the upstream region, exon 1 putative transit sequence, exon 1, and intron 1 regions of *Cpx2*, respectively (figure 4.2).

4.4.2 The Transcriptional Expression of *Cpx* Alleles

Individual plants carrying each of the *Cpx* mutant alleles were identified through the isolation of genomic DNA and PCR amplification of the locus as in the TUSC screening. Next, the genomic DNA was PCR-amplified with primers flanking the insertion site of each allele in order to detect the presence of a non-mutant copy of the

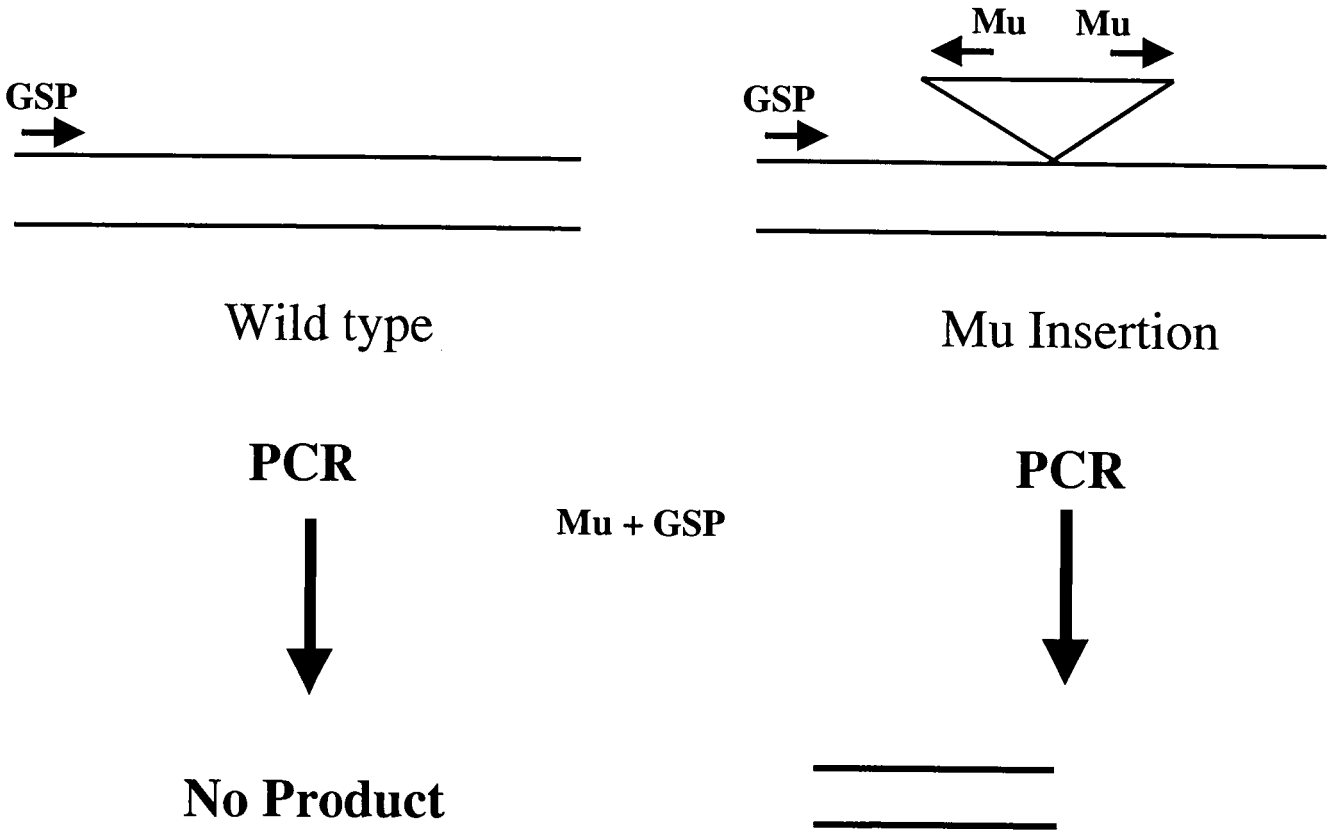

Figure 4.1 TUSC screening system. A mutator-insertion within a gene of interest can be identified through PCR amplification using a mutator-specific primer (Mu) and a gene-specific primer (GSP). Only the insertional alleles will support amplification using this primer set.

Table 4.1 The insertion points of the mutator alleles. *The precise insertional location is given as the number of bases from the first base of the putative start codon of each *Cpx* gene. Positive values indicate an insertion 3' of the start codon, negative values indicate an insertion 5' of the start codon.

allele	gene	location	insertion point*
cpx1-1	*Cpx1*	exon 1, within the putative transit peptide	+ 27
cpx1-116	*Cpx1*	intron1	+ 672
cpx2-104	*Cpx2*	5' untranslated region	- 3
cpx2-323	*Cpx2*	exon 1, within the putative transit peptide	+ 64
cpx2-578	*Cpx2*	exon 1	+ 366
cpx2-105	*Cpx2*	intron1	+ 729

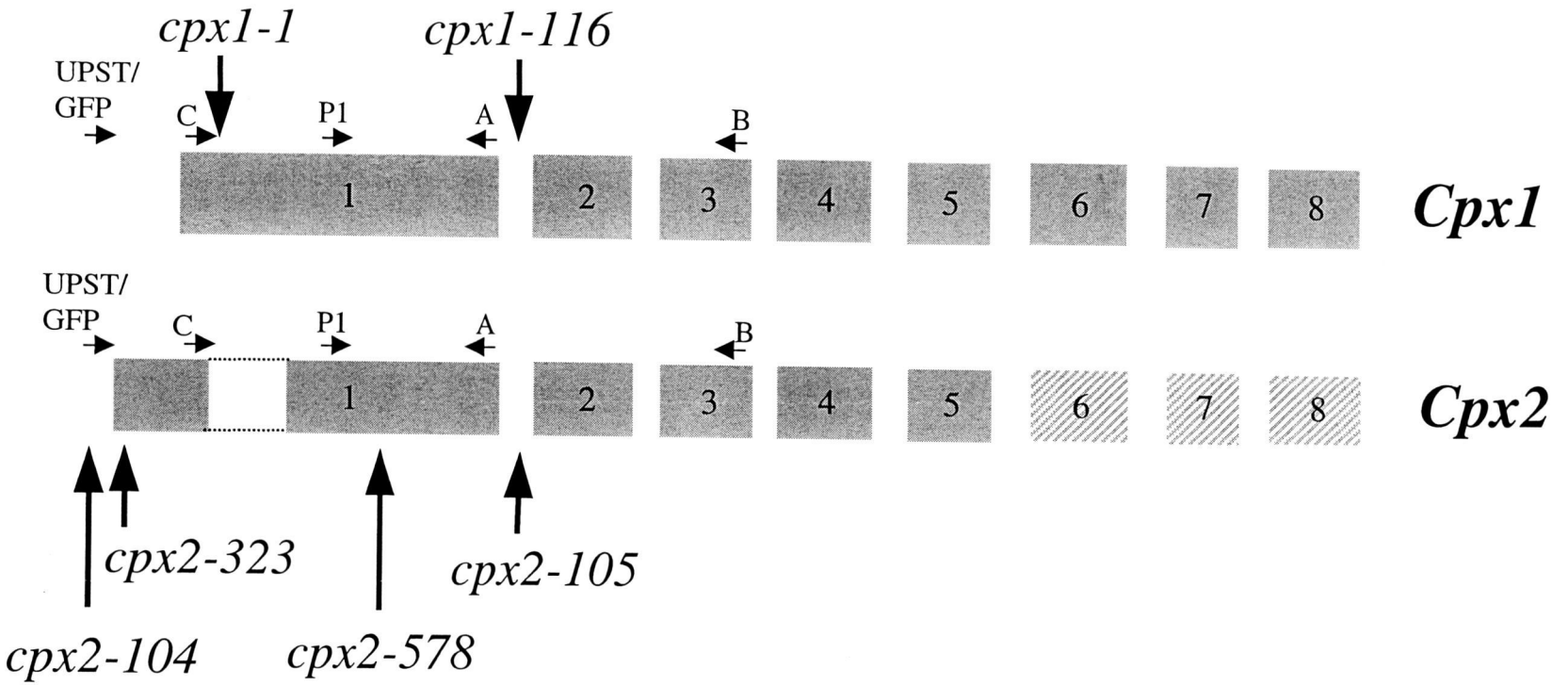

Figure 4.2 The mutator insertion alleles of *Cpx*. The relative sites of mutator insertion is diagrammed for each *Cpx* allele.The coding region of each *Cpx* gene is represented by a grey box, numbered according to the exon, and the hatched box represents the unsequenced region of *Cpx2*. The small horizontal arrows indicate primer annealing sites used in the PCR-based analysis.

Cpx gene in question. The plants chosen for analysis of the *Cpx* alleles *cpx1-1*, *cpx1-116*, and *cpx2-105* were all identified as heterozygous for the mutant alleles (data not shown). The plants studied for the *cpx2-323* and *cpx2-578* alleles were determined to be homozygotes (data not shown). The plant carrying for the *cpx2-104* allele could not be tested through PCR analysis because an appropriate upstream flanking primer has not been designed for the lack of sequence information across this region. Total RNA was extracted from each plant and from inbred line W-22 as a control. cDNA was produced from the RNA preparations and PCR-amplified independently with the following primer sets: UPST/GFP-Mu, B-Mu, C-B, and ZmacT1-ZmacT2. In all cases, the ZmacT1-ZmacT2 products were amplified, indicating that the control, actin cDNA was successfully reverse-transcribed from the RNA preparations (figure 4.3A). All cDNAs were PCR-amplified with primer set C-B (see figure 3.1) in order to detect the non-mutant allele of the *Cpx* gene in question or to detect a non-mutant transcript produced from a mutant allele (figure 4.3B). All cDNA preparations showed C-B PCR amplification of the non-mutant *Cpx*-gene products.

PCR products of sizes 729 bp and 429 bp were amplified from *Cpx* alleles *cpx1-1* and *cpx2-578*, respectively, using primer set B-Mu (figure 4.3C). None of the other alleles supported amplification from a transcriptional product in this assay. A second amplification product was detected in the *cpx2-578* sample; this product may have resulted from a second, minor priming site of one of the primers and is more noticeable under the less stringent amplification conditions of this assay. The *cpx1-1* and *cpx2-578* cDNA templates were amplified with the primer set C-Mu in order to detect the presence

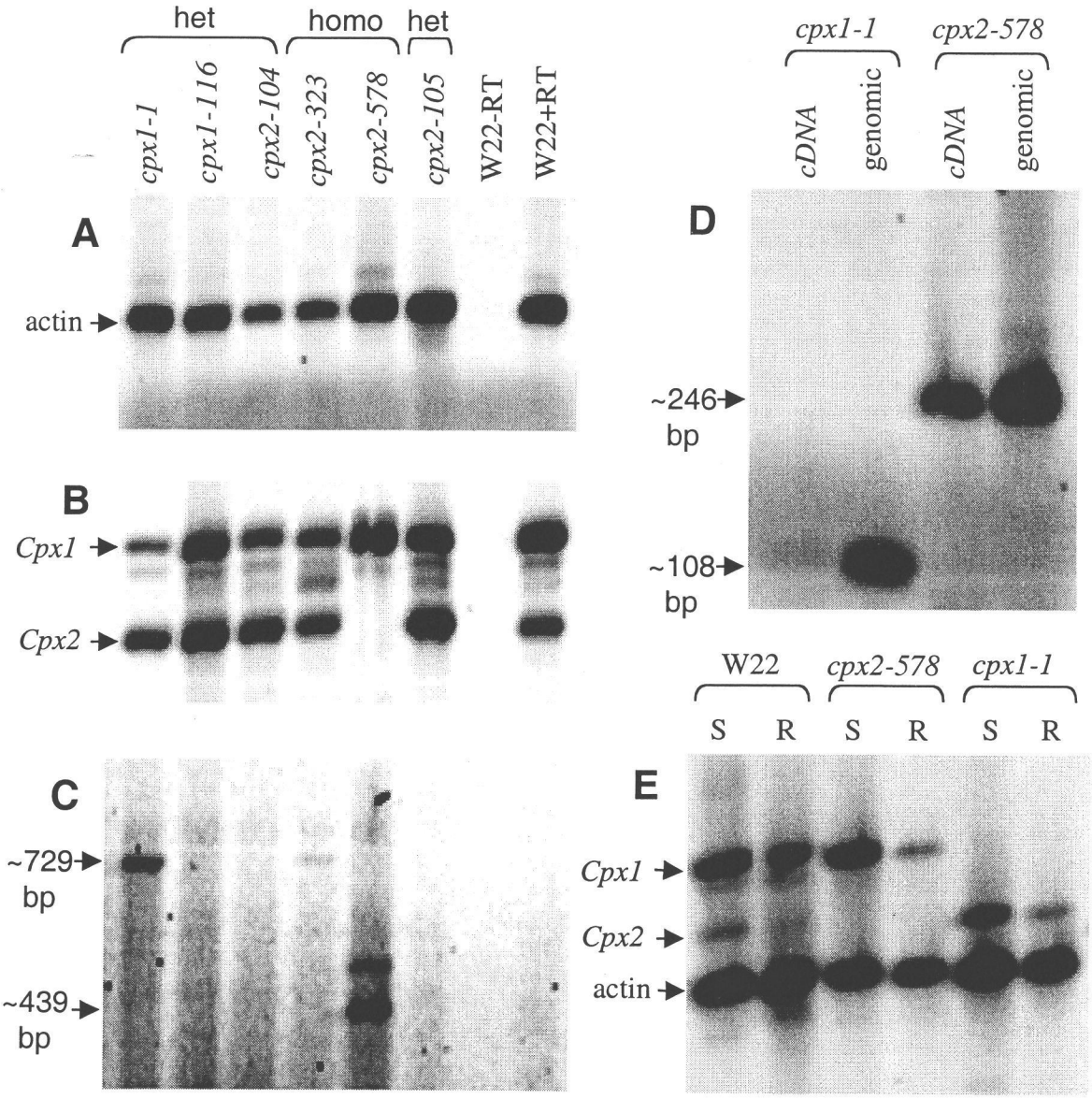

Figure 4.3 RT-PCR analysis of mutant *Cpx* alleles. Reverse images of ethidium bromide-stained amplification products separated by agarose gel electrophoresis are shown. Actin was amplified from each cDNA template as a control (A), W22+/-RT indicated the presence or absence of reverse transcriptase in the RT reaction with inbred line W-22 template. Non-mutant *Cpx* cDNAs (B) and mutant *Cpx* cDNAs (C) were amplified with primer sets C-B and Mu-B, respectively. Homozygotes (homo) and heterogygotes (het) are labelled. Mutant *Cpx* cDNAs were amplified upstream of the insertion site (D) using primer set Mu-C. Non-mutant *Cpx* and actin cDNAs were amplified from the shoots [S] and roots [R] of homozygous seedlings (E) using primer set C-B.

of a transcript 5' of the mutator insertion site (figure 4.3D). The amplifications were controlled with genomic DNA, which should produce identical-sized products. *cpx1-1* cDNA did not support amplification, but the amplification from *cpx2-578* cDNA resulted in a 246 bp product. The product size is consistent with amplification from the transcript upstream of the Mutator insertion site.

The *Cpx* alleles *cpx1-1* and *cpx2-578* were selected for further analysis because each have a Mutator insertion within the coding region of the mutated *Cpx* gene; therefore, each mutant would be less likely to express a functional product from the allele. Total RNA was extracted from seedlings homozygous for either *Cpx* allele, as determined through genomic PCR (data not shown) and subjected to RT-PCR analysis using primer set C-B, which spans both Mutator insertion sites. The *Cpx* alleles do not support the transcription of a normal-sized product (figure 4.3D). Thus, the mutant transcripts from each allele are disrupted by the Mutator element, indicating an inability to translate normal-sized proteins.

4.4.3 Description of Plants Segregating for Mutant Alleles

A preliminary phenotypic observation was made of the plants segregating for the newly-identified *Cpx* alleles. *cpx1-116* and *cpx2-105* were determined to be segregating heterozygotes and did not display any unusual characteristics beyond what may be expected of its parental Mutator line. Homozygous *cpx2-323* and *cpx2-578* plants did not display an unusual phenotype. The plant segregating for the *cpx2-104* allele also appears to be wild-type in phenotype. Germinated seedlings homozygous for *cpx2-578* do not have defective kernels and do produce root hairs, unlike *cpx1-1* homozygotes. Plants with

the genotypes *cpx1-1/+*, *cpx2-578/+* and *cpx1-1/+*, *cpx2-578/cpx2-578* appear normal and do reproduce. When plants with the genotype *cpx1-1/+*, *cpx2-578/cpx2-578* are inbred, defective kernels are present in approximately 1:3 ratio to normal kernels, as expected in seed segregating for the *cpx1-1* allele. On two occasions I attempted to germinate these defective kernels, putatively of the genotype *cpx1-1/cpx1-1*, *cpx2-578/cpx2-578*, and of a total of 50 seeds, none germinated. A well-controlled experiment was not executed due to the lack of mutant seed.

4.5 Discussion

The TUSC system enabled us to successfully identify 5 new mutant alleles of *Cpx*. Of these alleles, one has an insertion in *Cpx1*, the other four have insertions in *Cpx2*. Plants segregating for each of these alleles were harvested for RNA extractions and RT-PCR analysis. The *cpx2-323* allele does not produce a detectable Mutator-containing transcript from the mutant allele. The *cpx1-1* and *cpx2-578* alleles are transcribed, resulting in a mutant transcript containing Mutator sequence information.

Because the *cpx1-1* allele supported PCR amplification downstream of the insertion site but not upstream, transcription of this product may originate from within the Mutator element. Transcription would then be under the control of the new promoter and the expression of a product may be de-regulated from normal *Cpx1* expression. The site of Mutator insertion in *cpx1-1* is 15 bases downstream of the first methionine codon. If translation occurs from the mutant *cpx1-1* transcript, it would begin from within the Mutator element or farther downstream in the *Cpx* gene. A translational product produced from this transcript would originate at a methionine codon downstream of the Mutator

insertion site, producing a truncated product that is not expected to have a chloroplast localization.

The *cpx2-578* allele supported transcription both upstream and downstream of the Mutator insertion site, so that transcription probably occurs from the *Cpx2* promoter and results in a transcript that contains the entire Mutator sequence. This product does not contain a contiguous open-reading frame. There is also a possibility that translation may occur from the Mutator element or from a subsequent region in *Cpx2*. This product would not contain transit peptide information and would be missing some of the conserved region of exon 1, possibly disrupting CPX2 activity.

Plants heterozygous for any of the *Cpx* Mutator alleles appear normal, in contrast to the heterozygous lesion-mimic phenotype of *Les-22* mutants. A homozygous *Cpx2* mutant also has no phenotype, so the *Cpx2* deficiency may be complemented by as little as one copy of the *Cpx1* gene with no ill-effect on the plant. Other possibilities are that the mutant *Cpx2* transcript is sufficient to provide CPX2 activity even if the product is not mitochondrial-localized, or that CPX2 activity is dispensible. The reverse is not true, at least one *Cpx1* copy must encode a normal transcript or the plant has a yellow seedling lethal phenotype in inbred line H-99. This result demonstrates a redundancy in coprogen activity, but not a complete complementation in function.

The *cpx1-1* allele supports transcription of a mutant product that has a disruption in the chloroplast transit information. As a result, there should be no coprogen activity in the chloroplast and tetrapyrrolic intermediates, such as coproporphyrinogen III, would accumulate in the developing plastids of *cpx1-1* homozygotes. The CPX2 product is putatively localized in the mitochondria and it is not able to complement CPX1 activity in

order to produce heme and chlorophyll in the chloroplast, due to its probable localization. Thus, the yellow seedling phenotype of *cpx1-1* homozygotes in H-99, similar to the yellow seedling phenotype of *Les-22* homozygotes, represents an absence of chlorophyll production and perhaps a deficiency of chloroplast heme production as well.

The analysis of *Cpx1* and *Cpx2* mutant alleles provides an opportunity to separate the cellular functions of each gene product. Because the functions of CPX1 and CPX2 are not fully interchangeable, the role of each gene product must differ. *Cpx2* appears to be a non-essential gene or its function can be adequately supplied by *Cpx1* or by a *cpx2-578* truncated protein. One functional allele of *Cpx1* must be present for H-99 plants to develop beyond the seedling stage. Logically, the CPX1 product must perform an essential role in the cell, certainly it must be required at this stage for chlorophyll biosynthesis. Not so evident is the cellular heme requirement. Little is known about the maintenance of heme levels in the cell and the relative contributions of chloroplast-derived and mitochondrion-derived heme. Presumably, some level of heme must be required by plants in order to develop as far as the seedling stage. This may be evidenced by the inability of *cpx1-1*, *cpx2-578* double homozygotes to germinate. In *cpx1-1* homozygotes, this requirement may be met by CPX2 activity in the mitochondria, or by a mutant CPX1 product that is likely to be displaced from the chloroplast.

In *cpx2-578* homozygotes, chloroplast-localized heme production may be sufficient to generate the appropriate heme levels required by the plant. Alternatively, a mutant product from the *Cpx2* transcript could satisfy the CPX2 function. This option is unlikely because the Mutator allele disrupts the *Cpx2* open-reading frame within a highly conserved region. Another possibility is that protoporphyrinogen IX is translocated from

the chloroplast to the mitochondria, as is believed to occur in other plants. The *Cpx2* product may not be necessary under normal conditions, but could provide a detoxification function under conditions of oxidative stress. This leads me to suggest that while there may be transport of tetrapyrroles from the chloroplast to the mitochondria, the opposite may not be true; therefore, CPX2 activity cannot contribute to chlorophyll tetrapyrrole biosysthesis. Another factor may be the possibility of a significant transcriptional upregulation of *Cpx1*, but not *Cpx2*. The *Cpx1* gene may be influenced to produce greater quantities of coprogen activity, but *Cpx2* may only be expressed at a low, constitutive level. This could explain the ability of *Cpx1* to complement a *Cpx2* deficiency, but not the reverse. The differences between the phenotypes of *Cpx1* and *Cpx2* homozygous Mutator insertional mutants may be correlated with a divergence in the cellular role of each gene product.

4.6 Acknowledgements

I'd like to thank Kristine Hardeman for her role in the TUSC collaborative effort and Suzanna Hoheisel for her contribution in the identification of the *cpx2-578* mutant. Thanks also go to Pioneer HiBred International for their support in the TUSC identification of *Cpx* mutants.

5. Localization Studies of CPX in Maize

5.1 Abstract

The two *Cpx* genes of maize are transcriptionally expressed and both appear to encode functional coproporphyrinogen III oxidase (coprogen) enzymes. Analysis of the amino-terminal sequences of the predicted translational products of *Cpx1* and *Cpx2* suggests that they localize to different compartments. To test this hypothesis, a polyclonal antibody to tobacco coprogen was used to examine chloroplast and mitochondrial protein extracts from wild-type plants and plants homozygous for the *cpx2-578* allele, a Mutator transposon insertion into the *Cpx2* open-reading frame. In each fraction, except the mitochondria of the *cpx2-578* mutant, a 38 kDa protein was detected. The size of this protein corresponds to that expected for the processed products of *Cpx1* and *Cpx2*. Thus, the CPX2 product appears to be the source of the mitochondrial-localized 38 kDa protein. The putative targeting information of CPX2 was further analyzed by creating a fusion between the amino-terminal region of CPX2 and a GFP reporter. Upon biolistic transformation into maize leaf tissue, the CPX2::GFP fusion product was detected in discrete foci of 0.3-0.5 microns and exhibited a background cytoplasmic staining. The size and distribution of the foci resemble that a *bona fide* mitochondrial-localized control, COXIV::GFP (Kohler *et al* 1997). CPX1 is predicted to be chloroplast-localized and presumably plays a role in heme and chlorophyll biosynthesis in the chloroplast. The amino-terminal extension of CPX2 acts as a mitochondrial-targeting peptide and the CPX2 product appears to be localized within the mitochondria. The CPX2 enzyme is the

first mitochondrial-localized coprogen detected in a plant. I discuss the possible roles for this enzyme in heme biosynthesis and the detoxification of free tetrapyrroles.

5.2 Introduction

In angiosperms, the complete tetrapyrrole biosynthetic pathway occurs in the chloroplast (Fuesler, Castelfranco and Wong 1984; Tripathy and Rebeiz 1987). Only the terminal few enzymes in heme biosynthesis, protoporphyrinogen IX oxidase and ferrochelatase, have been identified in plant mitochondria (Jacobs and Jacobs 1987; Little and Jones 1976; Matringe *et al.* 1989a; Matringe *et al.* 1989b; Smith, Marsh and Elder 1993). It is believed that an intermediate of the tetrapyrrole biosynthetic pathway is translocated from the chloroplast to the mitochondria in order to supply an intermediate for heme biosynthesis (Smith, Marsh and Elder 1993). The candidate intermediate is protoporphyrinogen IX, the product of coproporphyrinogen III oxidase (coprogen) and substrate of protoporphyrinogen IX oxidase. The mechanism of transfer has not yet been defined.

In chapter 2, I hypothesized that a mitochondrial coprogen enzyme exists in maize. If this hypothesis is proven, then an earlier intermediate of the pathway may be translocated from the chloroplast to the mitochondria such that the substrate of coprogen, coproporphyrinogen III, is available for coprogen-catalyzed oxidations. In this section, I will present my analysis of the localization of the coprogen enzymes in maize (CPX) and discuss the proposed role of a mitochondrial-localized CPX product.

5.3 Methods

5.3.1 Protein Samples

Mitochondria and chloroplasts were isolated from 11-15 day-old seedlings of inbred line W-22 or plants homozygous for the *cpx2-578* mutation grown under 12h light/12h dark conditions at 25-29°C. Mitochondria were isolated according to a protocol developed for oats (Navarre and Wolpert 1995) that was slightly modified. Shoot tissue was homogenized in 300 mM sucrose, 30 mM MOPS (pH 7.5, KOH), 2 mM EGTA, 0.2% defatted BSA, and 1mM DTT, 5 ml per gram tissue. The filtered homogenate was centrifuged twice at 500g for 10 min, the pellets were used for subsequent chloroplast isolations, and the supernatant was used for mitochondrial isolations. The supernatant was centrifuged at 12,000g for 25 min and the pellet was collected and suspended in wash buffer (300 mM sucrose, 5 mM MOPS pH7.3 [TRIS, pH 8.0], 0.1 mM EGTA, 1mM DTT) and fractionated on Percoll gradients (Douce *et al* 1987). The gradient was centrifuged at 41,000g for 30 min and the mitochondrial layer was collected, diluted with 30 ml wash buffer, and centrifuged at 17,000g for 15 min. If necessary, the preparation was re-fractionated on a Percoll gradient and centrifuged at 17,000g again. This pellet was resuspended in 30 ml wash buffer and centrifuged at 12,000g for 15 min and the mitochondria-containing pellet was collected. Chloroplasts were isolated by resuspending the original pellets in wash buffer and overlaying the suspension on a 35%/70% continuous Percoll gradient. The chloroplast layer was collected, diluted in wash buffer as above, and centrifuged at 2,500g for 10 min and the chloroplast-containing pellet was collected. All centrifugations were performed in a Sorvall RC 5B Plus centrifuge

(DuPont) using an SS-34 rotor. Each protein sample came from a purified organelle preparation that was boiled for at least 4 min.

5.3.2 Western Analysis

Samples were loaded on a 12 or 14% denaturing acrylamide gel and electro-blotted according to the manufacturer's specifications (E-C Apparatus Corp.) onto a nitrocellulose membrane and blocked overnight in 1x PBS and 5% non-fat dehydrated milk. The anti-coprogen antiserum was generated against a truncated tobacco coprogen (CPO) expressed in *E.coli* (Kruse, Mock and Grimm 1995a; Kruse *et al.* 1995b). The mitochondrial-specific antibody was raised against malate dehydrogenase (MDH). The chloroplast-specific antibody was raised against photosystem *a* subunit D (PsaD). These antibodies were kindly provided to us by Bernhard Grimm (Institut für Pflanzengenetik und Kulturpflanzenforshung, Germany), Kathy Newton (University of Missouri), and Alice Barkan (University of Oregon), respectively. The α-CPO, α-MDH, and α-PsaD antibodies were used at 1:2000, 1:4000, and 1:1000 fold dilutions, respectively. The western blot detection was performed according to standard procedures (Ausubel *et al.* 1990). The primary antibodies were incubated for 2 hours and the goat α-rabbit alkaline-phosphatase conjugated secondary antibody (Southern Biotechnology Associates, Inc) was incubated for 1 hour and visualized using NBT/BCIP.

5.3.3 GFP Constructs, Transformation, and Analysis

The 5' region of the *Cpx2* gene was PCR amplified using the following primer set: CPX/UPST (see figure 4.2) and GFPx1 (5'-ATGGATCCGACGTCCTCGACGAAG-

3'). The amplified region encodes 139 amino acids of the amino-terminal region of CPX2, including the first 64 amino acids, which comprise the putative targeting sequence. The PCR conditions are as described in chapter 3, except for the following thermocycler parameters: 5 min at 94° (once), 1 min at 94°, 1 min at 48°, 2 min at 72° (five times), 1 min at 94°, 1 min at 62°, 2 min at 72° (30 times), and 5 min at 72°. The resulting fragment corresponding to the *Cpx2* gene was digested, gel-purified with the Quiex II kit (Qiagen), digested with BamHI, and extracted with phenol and chloroform. The isolated fragment was cloned into the BamHI site of pBCG, provided generously by M. Ivanchenko (Oregon State University), a GFP expression vector modified for use in plant systems (Chiu *et al* 1996). The clones were screened by restriction enzyme analysis for an insertion of the correct orientation such that a translational fusion was created between the open-reading frames of *Cpx2* and *gfp*. The candidate CPX2::GFP construct was verified through sequence analysis by the Central Services Laboratory at Oregon State University. Material for transformation was prepared by dissecting unemerged leaves from one month-old W-22 (Bilang and Bogorad 1996). The leaf tissue was cultured on MS plates overnight and kept in the dark to prevent greening. The tissue was transformed via the biolistic method using a helium-driven PDS-1000/He system (BioRad), following the manufacturer's bulletin (Heiser 1992). The tissue was observed roughly 48 hours after bombardment with a Leica confocal laser scanning microscope and GFP was detected using standard fluorscein filters. Optical sections were taken at 1 micron intervals. The images were enhanced with pseudo-color on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of

Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) and processed with Adobe Photoshop (Adobe Systems Inc.).

5.4 Results

5.4.1 Immunological Detection of CPX

Based on their putative amino-terminal targeting peptides, CPX1 and CPX2 could be directed to different cellular compartments. The *Cpx1* transcript encodes a fairly canonical chloroplast transit peptide. This is missing from the *Cpx2* gene, whose 5' sequence appears more similar to a mitochondrial targeting peptide. To look for the presence of CPX products in these compartments, western blot analysis was performed. Antiserum to tobacco coproporphyrinogen III oxidase was used to detect CPX proteins in extracts of isolated chloroplasts and mitochondria. To verify protein extracts (figure 5.1A) and control for cross-contamination, the same samples were tested using chloroplast- and mitochondrial-specific antisera. The purified mitochondrial samples contained the mitochondria-specific antigen, MDH; the chloroplast preparations were not detectably contaminated with this antigen (figure 5.1B). The purified chloroplast samples contained the chloroplast-specific antigen, PsaD; whereas, the mitochondrial samples did not have a detectable level of this antigen (figure 5.1C).

For CPX localization analysis, chloroplasts and mitochondria were isolated from the seedling shoots of the wild-type inbred line W-22 and from *cpx2-578* homozygotes, a mutator transposon insertion allele of *Cpx2* (for details, see chapter 4). The chloroplast and mitochondrial proteins from each genotype were separated on a denaturing SDS-acrylamide gel and western blot analysis was performed. In samples from the wild-type

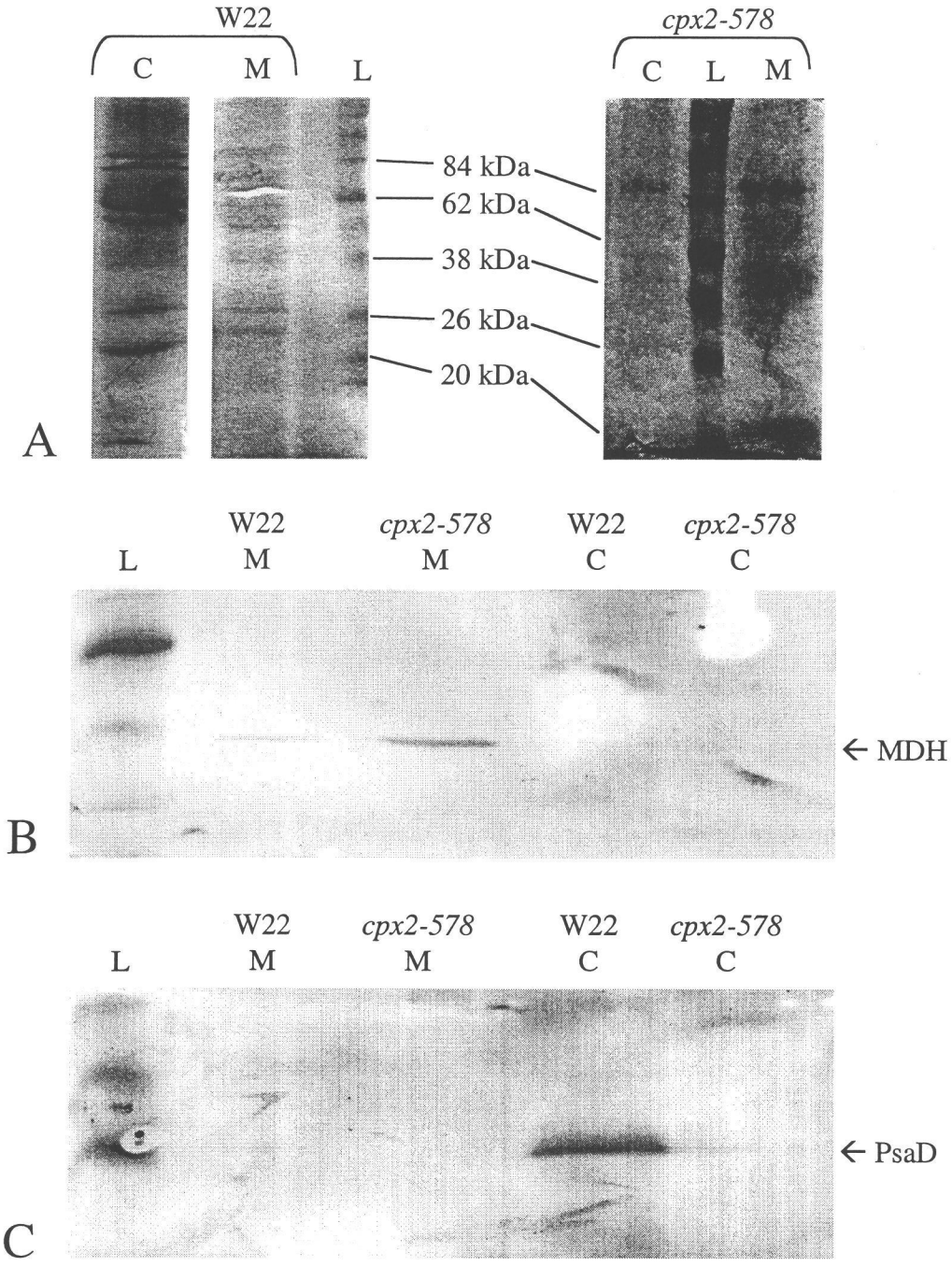

Figure 5.1 Protein controls for immunological assay. The mitochondrial (M) and chloroplast (C) protein preparations were run on an acrylamide gel and stained with coumassie blue (A). The amount of protein in the C and M lanes of W22 and that of *cpx2-578* are 17 μg, 8 μg, 7 μg, and 10 μg, respectively. The samples were western blotted and detected with α-MDH (B) and α-PsaD (C) antisera. Figures B and C are different halves of the same protein gel and contain the following amounts of protein in each lane: 8 μg, 16 μg, 17 μg, and 4 μg, in that order. The protein ladder is represented by 'L'.

W-22, the anti-CPX antisera recognized a 37.5 kDa protein in the mitochondrial (figure 5.2B) and chloroplast (figure 5.2C) samples. An additional band of 26.8 kDa was also observed in the mitochondrial sample. A 37.5 kDa chloroplast protein was detected in samples from the *cpx2-578* mutant, but the mitochondrial samples contained the 26.8 kDa band, not the 37.5 kDa protein. The data indicate that CPX1 is specifically localized to the chloroplast, and CPX2 to the mitochndria. The 26.8 kDa protein recognized by the antisera in the mitochondrial samples is much smaller than expected for the CPX protein. Whether it is a breakdown product of CPX, or an antigenically similar protein is unknown.

5.4.2 The Localization of CPX2::GFP

The mitochondrial localization of CPX2 is presumably due to targeting information encoded in the 5' sequence that differs between the two *Cpx* genes. To test whether this sequence is sufficient for mitochondrial targeting, fusion constructs were made with the Green-Fluorescent Protein (GFP), a single polypeptide that can be visualized directly due to its fluorescent properties (Chiu *et al.* 1996). A CPX2::GFP construct was made that encodes the first 139 amino acids of the presumed amino-terminal region of CPX2 fused in frame to the entire GFP open-reading frame (figure 5.3). The 35S CaMV promoter drives transcription of the construct and either the upstream or downstream methionine codon of *Cpx2* could act as the translational start of the fusion protein (see figure 2.2). The vector also contains the Adh-1 intron, which increases the expression of the construct, and the nopaline synthetase transciptional terminator. This construct was transformed into etiolated W-22 seedling leaf tissue using

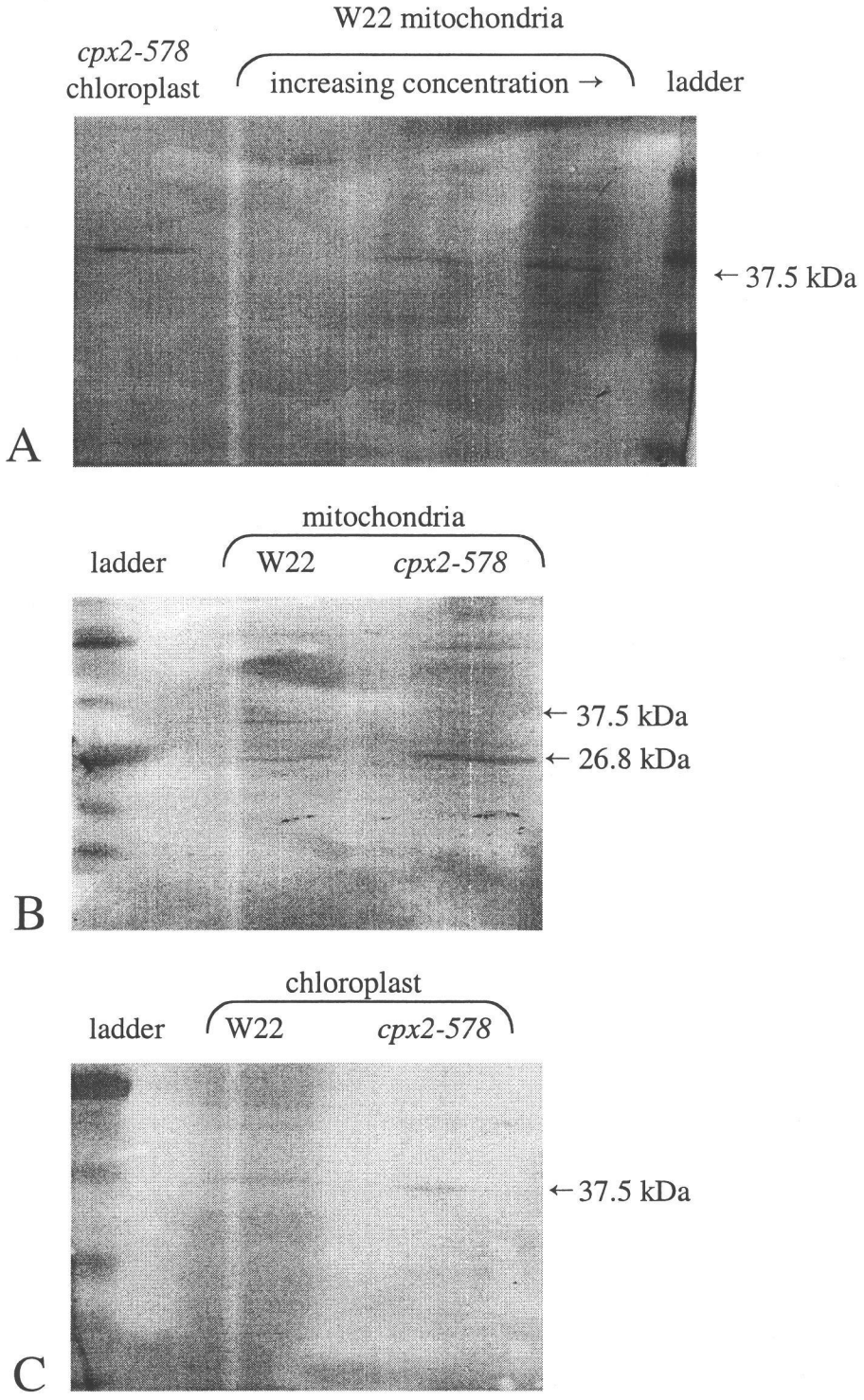

Figure 5.2 Immunological detection assay. Chloroplast and mitochondrial protein preparations were western blotted and detected with antisera to tobacco coprogen. The relative sizes of the chloroplast and mitochondrial proteins are compared (A). The mitochondrial (B) and chloroplast (C) proteins of W-22 and *cpx2-578* plants are compared individually, the relative amounts of protein are the same as in figure 5.1, images B and C.

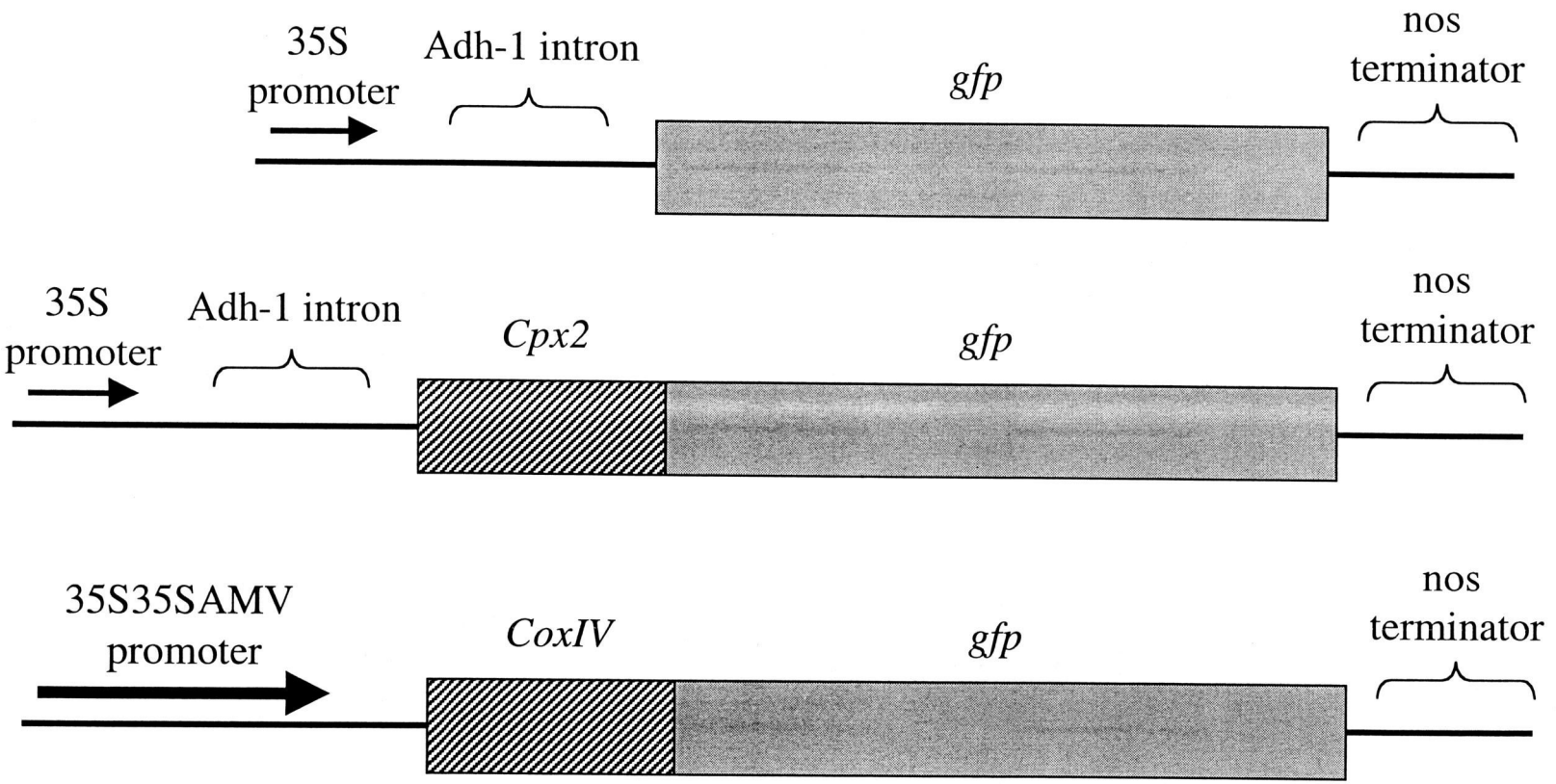

Figure 5.3 Schematic of the GFP constructs. The 5' regions of *cpx2* and *CoxIV* were cloned into a vector that constitutively expresses GFP. The boxed regions represent the coding sequences of the construct.

gene gun bombardment. After approximately 48 hours, the leaf tissue was observed under a fluorescence microscope in order to detect the location of the CPX2::GFP fusion protein and confocal images were taken. The GPF-encoding vector was used as a negative control, demonstrating general cytoplasmic staining. The 35S35SAMV::COXIV::mGFP4 construct was used as a positive control. COXIV is a mitochondrial-localized cytochrome that functions in the respiratory electron transport chain. The product of this construct specifically localizes GFP to the mitochondria and is under the control of a much stronger promoter, the binary 35S35SAMV promoter (Datla *et al* 1993). The fluorescent images are shown in pseudo-color; yellow represents high intensity fluorescence and red tones correspond to a low level of fluorescence.

The GFP vector control expresses GFP continuously throughout the cytoplasm in a relatively even distribution (figure 5.4A). CPX2::GFP has a distinct localization pattern with a particulate distribution of fluorescent foci as well as a background fluorescence distributed throughout the cytoplasm (figure 5.4B). The 35S35SAMV::COXIV::mGFP4 positive control localizes GFP exclusively in particulate foci that have been previously identified as mitochondria (Kohler *et al.* 1997)(figure 5.4C). The fluorescent foci of the CPX2 and COXIV constructs are both approximately 0.3-0.5 microns in size, the COXIV foci appear brighter and in some cases slightly larger than that of CPX2 in similar-sized cells. In each transformation, the neighboring untransformed cells show negative fluorescence under the imaging conditions used. Optical sections were taken of a CPX2::GFP transformed cell in order to visualize the 3-dimentional distribution of the fusion product within the cell (figure 5.5). The particulate foci are evident throughout the cell and appear to be excluded from the nucleus and vacuoles. The higher distribution of

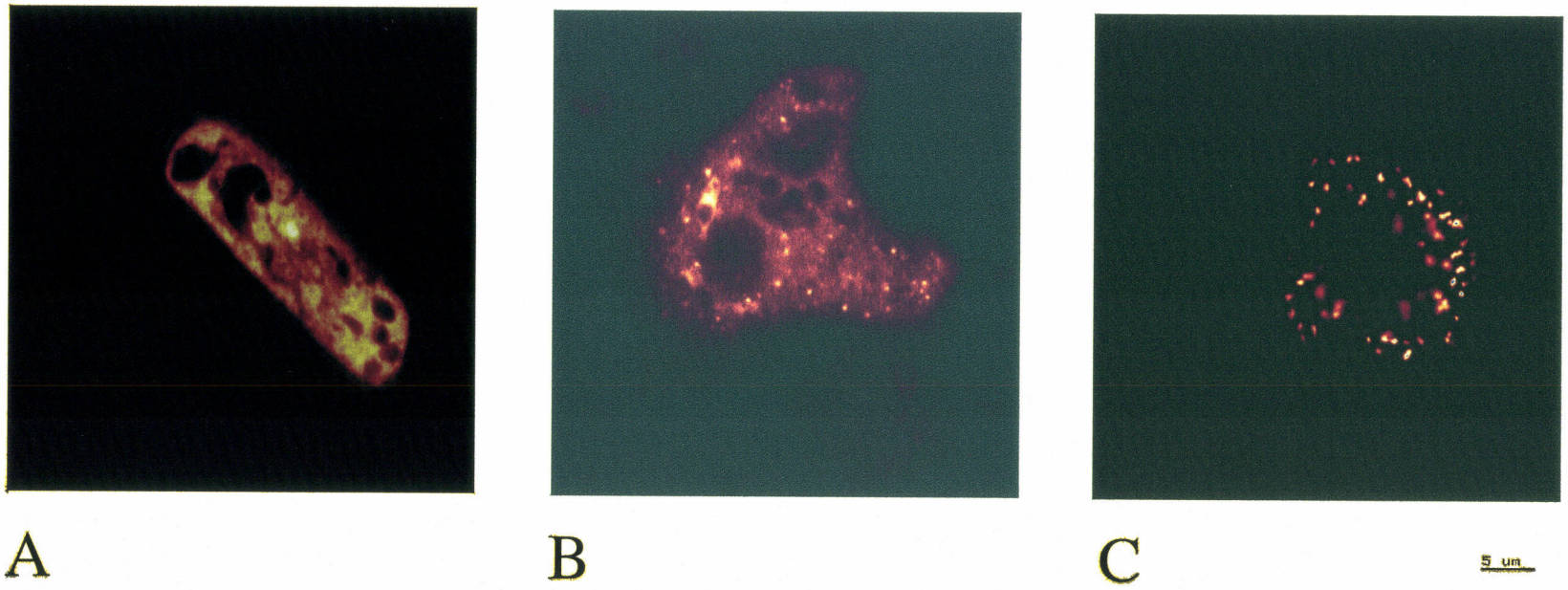

Figure 5.4 GFP detection. The color-enhanced fluorescent images of maize leaf epidermal cells transformed with the following constructs are shown: GFP (A), CPX2::GFP (B), and 35S35SAMV::COXIV::mGFP4 (C).

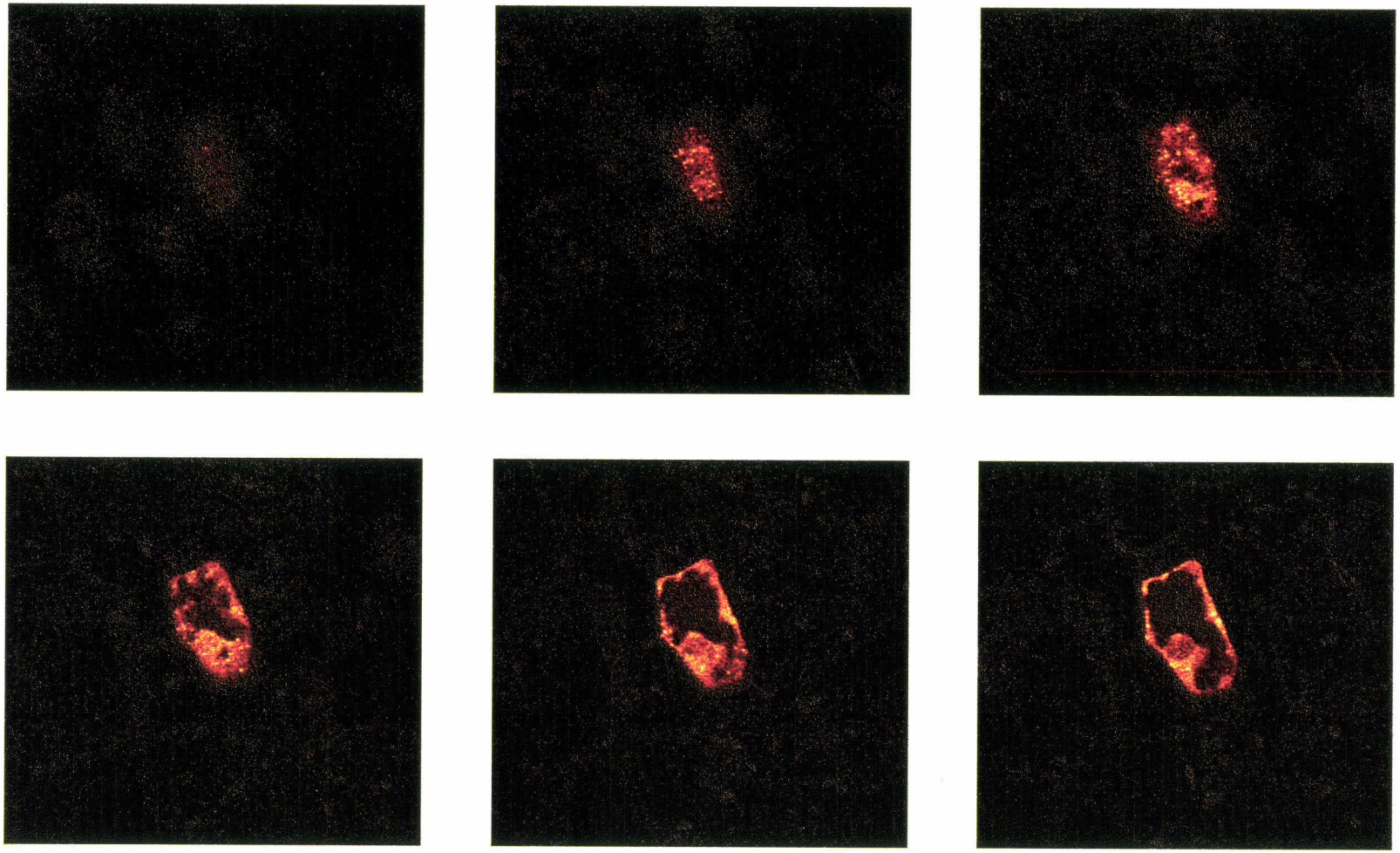

Figure 5.5 Optical sectioning of a CPX2::GFP transformed cell. The sections were taken at 1 micron intervals with a confocal microscope.

foci towards the upper sections of the cell may be representative of the natural distribution of the foci or could be the result of a decrease resolution of the lower optical sections. The distribution of foci resembles optical sections taken of CPXIV::GFP transformants (data not shown). Occasionally, a bright, irregularly-shaped patch of fluorescence is detected in a CPX2::GFP transformed cell, the nature of this patch is undetermined.

5.5 Discussion

Proteins with CPO-like antigens were detected in both the chloroplasts and mitochondria of W-22 seedlings. There does not appear to be a significant amount of chloroplast contamination in the mitochondrial samples. The *cpx2-578* mutation specifically disrupts the open-reading frame of *Cpx2* (see chapter 4); it is unlikely that this gene encodes an active CPX enzyme. Because normal CPX2 expression is disrupted, the mitochondria of the mutant are not expected to contain a CPX enzyme. The data supports this prediction; a coprogen-like protein was identified in the isolated chloroplasts of the *cpx2-578* mutant, but not in the mitochondria. This result is also consistent with the fact that *cpx2-578* has wild-type copies of the gene encoding CPX1, the putative chloroplast-localized enzyme, and the chloroplast-localized coprogen-like protein is present in the *cpx2-578* mutant. The data is preliminary, and should be subject to more rigorous experimentation. The coprogen-specific antisera picks up multiple bands and a high background and should be purified for a more exclusive detection of coprogen antigens. The relative protein concentrations should be adjusted and the samples should be more carefully treated to avoid protein degredation.

The CPX2::GFP-translational fusions altered the localization of GFP. CPX2::GFP was identified in 0.3-0.5 micron foci, consistent with the size and distribution of mitochondria. The slight size variation between the foci of CPX2::GFP and that of the COXIV fusion protein could be the result of the much stronger signal of COXIV as a result of the highly expressed 35S35SAMV promoter. The amino-terminus of CPX2 appears to direct GFP to the mitochondria. It is likely that translation begins at the upstream methionine codon (figures 2.2 and 2.3), generating an amino-terminal extension that acts as a mitochondria transit peptide. Translation also may occur in this system at the downstream methionine codon, as detected by the background cytoplasmic staining. It is possible that translation could start at this methionine codon *in vivo*, or that this expression is an artifact of the construct. It is unlikely that the foci represent plastid-localization because the relative size of these organelles are at least 5-fold larger in diameter. Another possibility is that the foci are cytoplasmic aggregates of the GFP fusion product. The consistent size range of these foci is less likely to occur from a random clumping of fluorescent product, although this does not disprove the possibility. A more definitive experiment is underway to positively identify mitochondria immunologically, detecting them with a different fluorescent marker. Both the immunological and cytological evidence shown here supports the theory that CPX1 is chloroplast-localized and CPX2 is mitochondrial-localized.

The CPX2 product is the first plant mitochondrial-localized CPX enzyme identified. Because the catalytic region of this product is so highly conserved with other coprogen enzymes, is it likely to function as a CPX enzyme. The mystery is whether there is substrate, coproporphyrinogen III, present in the mitochondria of maize. If

coproporphyrinogen III is present in the mitochondria, its source could be generated in several ways. One possibility is that more of the tetrapyrrole biosynthetic pathway occurs in the mitochondria of maize than in the other plants that have been studied. Perhaps coproporphyrinogen III or an earlier precursor moves from the chloroplast to the mitochondrion to act as a substrate for heme biosynthesis. It is not known whether any of the preceding precursors or enzymes are present in the mitochondria of maize. It is possible that the entire biosynthetic pathway occurs there. A second possibility is that a mitochondrial CPX enzyme could act to detoxify coproporphyrinogen III 'contamination' in the mitochondrion. Little is known about how an intermediate of tetrapyrrole biosynthesis is translocated from the chloroplast to the mitochondrion, but if the translocated intermediate is protoporphyrinogen IX, as expected, it is possible that other very similar intermediates, such as coproporphyrinogen III, are also translocated at some level. Coproporphyrinogen III is a phototoxic intermediate, detoxification of this porphyrin could provide a substantial benefit to maintaining a low level of mitochondrial oxidative damage. This notion is supported by studies of a mutation in the preceding enzyme, uroporphyrinogen III decarboxylase, in maize. This mutation appears to have a degradative effect on the mitochondrion (G.S. Johal, personal communication). This observation could correlate with a build-up of uroporphyrin III in the chloroplast, the translocation of this precursor at a higher rate to the mitochondrion, and a phototoxic effect on the mitochondrion as the result of this accumulation. Further study is required to investigate these possibilities.

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6.0 Conclusions

In the preceding chapters I have presented my analysis of the maize *Cpx* genes, their expression, and the localization of their products. In addition, I have identified four novel Mutator insertional mutant alleles of the *Cpx* genes *(cpx1-116*, *cpx2-104*, *cpx2-323*, and *cpx2-105)* and performed a preliminary characterization of these and two other mutant alleles *(cpx1-1* and *cpx2-578)*. In the following chapter, I would like to summarize these findings, presenting them in relation to my theory that the two *Cpx* genes of maize have specific and independent roles within the cell.

The sequences of two maize genes putatively encoding CPX were compared and determined to have a very conserved open-reading frame. In fact, the maize genes are more homologous to one another than to that of other characterized *Cpx* genes and are located in syntenous regions of the genome. It is probable that the two genes originated from a duplication event during the speciation of maize. The translated products of these genes closely adhere to three invariant regions within the C-terminal region of known CPX enzymes, domains that are believed to contain the catalytic region of CPX. This data is consistent with the possibility that both genes encode active CPX enzymes that are likely to catalyze identical reactions. In other words, the existence of two CPX-encoding genes represents a redundancy in the maize genome.

Two significant differences between the *Cpx* genes are the following: the presence of a 150 base pair deletion in the sequence of *Cpx2*, relative to *Cpx1*, and the presence of an in-frame methionine codon 213 base pairs 5' of the conserved methionine start codon of *Cpx2*. These differences are likely to have resulted from a series of deletions in one of the duplicated *Cpx* genes, resulting in *Cpx2*. The translated products

of these genes have very different amino-terminal regions, altering the putative transit peptide information. Analysis of these regions predicted CPX1 as chloroplast-localized and CPX2 as mitochondrial-localized. In a set of preliminary experiments, both the western blot analysis and GFP-localization study identified CPX2 as mitochondrial. By default, CPX1 was predicted to be chloroplast-localized. The putative difference in compartmentalization of each CPX enzyme represents a dramatic divergence between the gene products and supports the possibility that each enzyme may have a specific role in that location. Heme and chlorophyll biosynthesis occurs within the chloroplast, and the final stages of heme synthesis also occur within the mitochondria of plants. By inference, CPX1 must play a role in heme and chlorophyll biosynthesis and CPX2 may play a role in heme biosynthesis. Thus, while the two enzymes may catalyze an identical reaction, their localization predicts that CPX1 will promote synthesis of heme and chlorophyll, while CPX2 will contribute only to the mitochondrial portion of heme synthesis.

Another difference between the *Cpx* genes is their transcriptional expression pattern. Although each gene appears to be transcribed in all tissues, the relative expression level of *Cpx1* is modulated. *Cpx1* is upregulated in shoot tissue, which corresponds nicely with its putative role in chlorophyll biosynthesis. The lower level of *Cpx1* expression in root tissue and the relatively constant expression level of *Cpx2* are consistent with a lower constitutive expression that may be associated with heme biosynthesis. These data support the possibility that coprogen activity in the two compartments responds to different needs for tetrapyrroles.

Plants homozygous for the *cpx1-1* or *cpx2-578* alleles have very different phenotypes from one another. The *cpx1-1* yellow seedlings are deficient in chlorophyll

production and they become necrotic even when seedling growth is supported in tissue culture medium (K. Hardeman, unpublished data). In contrast, *cpx2-578* plants are normal in appearance. This may be due to a complementation of the *Cpx2* deficiency by *Cpx1*, which can produce protoporphyrinogen IX in the chloroplast. These data are also consistent with the *Cpx* genes having individual, yet overlapping functions within the cell.

In summary, despite that fact that the genes are likely to encode very similar enzymes, each enzyme has a specific cellular localization, transcriptional expression pattern, and mutant phenotype. The roles of each enzyme are independent, but with a potential functional overlap. The evidence and conclusions presented here differs significantly from what is known about tetrapyrrole biosynthesis in plants. No other plant has been characterized as containing two CPX enzymes. None of the identified CPX enzymes in plants have been determined to be mitochondria-localized. The mutant alleles of the *Cpx* genes are the only known mutations specifically disrupting enzymes of porphyrin biosynthesis in plants, apart from the *Les-22* mutant of maize (Hu *et al.* 1998).

The implication of these findings is that tetrapyrrole biosynthesis in maize differs from that in other plants. The presence of a mitochondrial-localized CPX enzyme supports the possibility that more of the heme biosynthetic pathway takes place in the mitochondria of maize than previously known, perhaps even the entire pathway. Further study of the porphyrin content of maize mitochondria would elucidate this theory. The separation of mitochondrial heme biosynthesis from chloroplast tetrapyrrole biosynthesis provides the opportunity for independent regulation of each. Because heme has been characterized as a negative regulator of the pathway, the separation of cellular heme

production from chlorophyll production by compartmentalization would remove feedback inhibition from chlorophyll biosynthesis. The de-regulated pathway of chlorophyll biosynthesis could provide an avenue for the high level of chlorophyll production in photosynthetic tissue. Chloroplasts are also capable of heme biosynthesis, perhaps it is produced less at this location during the up-regulation of chlorophyll biosynthesis or excess heme is effectively transported out of the chloroplast during this time. A second possibility is that coproporphyrinogen III is present in the mitochondria as a contaminant and CPX acts to detoxify its photoreactive effect. A maize mutation in uroporphyrinogen III decarboxylase, the preceding enzyme of tetrapyrrole biosynthesis, has a degredative effect on mitochondria (G.H. Johal, personal communication), suggesting that the phototoxic precursors of protoporphyrinogen IX can be transported to the mitochondria. A mitochondrial-localized CPX enzyme may eliminate the accumulation of coproporphyrinogen III, preventing oxidative damage from occurring.

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