Victoria blight of oats is caused by the fungus *Cochliobolus victoriae*. This fungus is pathogenic due to its ability to produce the host-selective toxin victorin. Previously, a 100-kD protein that binds victorin in vivo only in susceptible genotypes was identified as the P protein of the glycine decarboxylase complex (GDC). Victorin is a potent in vivo inhibitor of GDC. Leaf slices pretreated with victorin displayed an effective concentration for 50% inhibition (EC$_{50}$) of 81 pM for GDA. Victorin inhibited the glycine-bicarbonate exchange reaction in vitro with an EC$_{50}$ of 23 µM. We also identified a 15-kD mitochondrial protein in susceptible and resistant genotypes that bound victorin. Amino acid sequence analysis indicated this protein is the H protein component of the GDC. Thus, victorin specifically binds to two components of the GDC. Victorin had no detectable effect on GDC in isolated mitochondria, apparently due to the inability of isolated mitochondria to import victorin. The interaction of victorin with the GDC may be central to victorin's mode of action. Supporting this observation is the finding that CO$_2$ gives partial protection against victorin. Elevated CO$_2$ is known to ameliorate the effect of GDC inhibition. Victorin treated plants incubated in the light develop more severe symptoms than dark-incubated plants. Victorin appears to induce a plant-wide signal transduction cascade, resulting in diverse effects. Victorin induces specific proteolytic cleavage of the Rubisco large subunit (LSU). Leaf slices incubated
with victorin for 4 hours in the dark accumulate a form of LSU which is cleaved after the N-terminal lysine 14. LSU cleavage in leaf slices is prevented by the protease inhibitors E-64 and calpeptin. LaCl₃ prevents this cleavage of LSU and LaCl₃ also confers complete protection against victorin at the whole plant level. Victorin also causes lipid peroxidation as measured by MDA accumulation. DNA laddering is seen in leaves after 3 hr treatment with toxin. The ethylene inhibitors AOA and STS give significant protection against victorin at the whole plant level, and also prevent LSU cleavage.
Doctor of Philosophy thesis of Duroy A. Navarre presented on January 22, 1997

APPROVED: 

Major Professor, representing Botany and Plant Pathology

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

Duroy A. Navarre, Author
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CHAPTER 1

INTRODUCTION

Host Selective Toxins

Progress towards understanding the molecular and biochemical basis of plant disease resistance has proceeded at a rapid pace during the last few years. One of the major goals in this research field, the cloning of disease resistance genes, has finally been achieved and at least 5 resistance genes have been cloned (reviewed by Bent, 1996). However, with one exception, the role of these cloned disease resistance genes is not yet understood. What can be gleaned about these genes is that they are apparently components of a complex signal transduction chain. For example, the N gene, which is involved in tobacco resistance to TMV, was cloned and found to have homology to the Drosophila Toll gene and mammalian interleukin-1 receptor gene which suggests that the N gene could play a similarly complex role in plants (Whitham et al., 1994). Indeed, many of the recent advances have led to more questions than answers, uncovering an even higher degree of complexity in plant defense responses than had been anticipated.

Host-pathogen interactions are complex by nature and determining the primary causes of disease resistance or susceptibility is usually difficult to resolve from secondary events occurring in both the host and pathogen. Host-selective toxins simplify the study of such interactions because the toxin is the primary determinant of both the pathogenicity and
host-range of the pathogen. All known host-selective toxins are produced by fungi and are usually toxic only to plants that are also susceptible to the fungus which produces the toxin. Furthermore, fungi which do not produce the toxin are not pathogenic, or are weakly virulent. Host-selective toxins (HSTs) also generally elicit the same symptoms in the plant as inoculation of the plant with the toxin-producing fungus. Thus, HSTs obviate the need to inoculate a plant with a living organism and, consequently, analyzing the plant disease response is not complicated by the presence of a pathogen. The plant response to HSTs typically occurs within hours of treatment, as rather than days or weeks as is usually required with a living pathogen. Another advantage of HSTs is that they provide a discrete biochemical starting point to investigate the plant response.

Clearly HSTs offer many advantages. Not surprisingly, they have provided novel insights into the plant disease response. The first plant disease resistance gene cloned was the Hml gene of maize which encodes a protein which inactivates the HST produced by Cochliobolus carbonum (Johal and Briggs, 1992). One of the first reports of apoptosis in the plant disease response resulted from analysis of the response of tomato to AAL toxin, a HST (Wang et al., 1996). The first identification of the molecular basis of disease susceptibility in a host-pathogen interaction resulted from work with T-toxin and maize (Dewey et al., 1988). Another novel mechanism of pathogenicity was uncovered upon finding that HC toxin, another HST, apparently prevents maize from mounting a defense response by inhibiting histone deacetylase, an effect which has been speculated to prevent maize from inducing the transcription of plant defense genes, apparently by keeping chromatin in a state which prevents transcription (Brosch et al., 1995). Thus, HSTs have proven to be valuable tools in the study of the plant disease response.
Ultimately, all of this work can be traced back to the discovery of victorin, the prototypical HST, which gave rise to the concept and study of host-selective toxins.

**History of Victorin Research**

Victoria blight disease of oats, caused by the fungus *Cochliobolus victoriae*, was first described by Meehan and Murphy in 1946. Reports in the 1940s of the involvement of a toxin in Victoria blight disease provided the major impetus for early research into the role of host-selective toxins in plant disease. Only oat lines containing Victoria-type resistance to crown rust are susceptible to *C. victoriae*. The oat variety, Victoria, was introduced to the USA from Uruguay in 1927 and used for the breeding of varieties with “outstanding” crown rust resistance (Litzenberger, 1949). Plants derived from the Victoria variety underwent a hypersensitive response when challenged with the then prevalent rust races. By 1945, 75% of the USA oat acreage contained plants derived from the Victoria variety. Within two years of its discovery, Victoria blight had become a major oat disease due to the extensive acreage containing Victoria-derived plants and had extended into Canada (Hamilton and Broadfoot, 1947). The novel discovery was made that cell-free *C. victoriae* culture filtrates contained a toxic substance that not only reproduced the symptoms of the fungus on susceptible plants, but also had the same specificity as the pathogen. The culture filtrates had no effect on oats lacking victoria-type resistance (Meehan and Murphy, 1947). This toxin also had no effect on any other plants tested nor on various microorganisms (Luke and Wheeler, 1955). The potency of the toxin, demonstrated by the finding that culture filtrates diluted one-million-fold inhibited root growth 50% in sensitive varieties, generated additional interest in this host-
parasite interaction. The toxin was partially purified and given the name victorin (Wheeler and Luke, 1954). Sensitivity to victorin and, consequently, susceptibility to *C. victoriae*, is controlled by a single dominant nuclear gene (*Vb*) (Litzenberger, 1949). Hence, analysis of this host-parasite interaction is simplified to an analysis of the interaction of a single fungal metabolite with the product of a single plant gene.

Characterization of the effect of victorin on plants continued. In 1959 it was shown that victorin causes a respiratory burst in susceptible, but not resistant plants (Romanko, 1959). Wheeler and Black in 1962 reported that victorin caused a change in cell permeability and greatly increased cation leakage. This change in cell permeability correlated strongly with the wilting, the primary victorin symptom. This led victorin researchers to start considering the plasma membrane as the site of action. Further support for this viewpoint was obtained in 1966, when electron microscopy revealed separation of the plasma membrane from the cell wall and the appearance of a dark-staining material between the cell wall and cell membrane (Luke et al., 1966b). Also in 1966, victorin was found to cause a loss of respiratory control in mitochondria isolated from victorin-treated tissue, but victorin had no effect when applied directly to isolated mitochondria (Wheeler and Hanchey, 1966). They concluded that victorin caused a loss of mitochondrial integrity, but indirectly, since victorin had no effect on isolated mitochondria. Until this point, the prevalent opinion about the mode of action of victorin had been that the primary effect of victorin was an uncoupling of oxidation from phosphorylation. By 1968 the hypothesis that the plasma membrane was the site of action was firmly entrenched and researchers speculated that resistant plants lacked a cell membrane receptor that susceptible plants possessed (Samaddar and Scheffer, 1968). At
this time, the two major research programs dominating the victorin research field were headed by Wheeler and Scheffer, respectively, and they were fierce competitors, with one group often publishing results which refuted the previously published results of the other group. In this instance, Wheeler quickly disputed the claim that resistant plants lack a receptor, citing the fact that when resistant plants are treated with very high doses of victorin, they undergo increased respiration, electrolyte leakage and decreased root growth and this effect did not occur if plants were treated with deactivated toxin (Wheeler and Doupnik, 1969). Possibly, Wheeler's data was discounted because he had to use very concentrated amounts of relatively crude (by modern standards) toxin preparations and the effects he observed could have been due to impurities. The opinion that resistant plants lacked a receptor became prevalent, often heard voiced 20 years later, despite any real evidence. Turner (1972) found that victorin decreases transpiration and causes stomates to close and speculated the closure could be a result of victorin's known ability to cause a K+ efflux. In 1973, Gardner and Scheffer reported that a 12 hr pretreatment with 5 μg/ml cycloheximide decreased sensitivity to toxin by 70-90% and cited this as supporting evidence that victorin interacts with specific protein receptors. Luke and Barnett (1974) observed in 1974 that susceptible cultivars grown in a solution containing calcium were more sensitive to toxin than plants grown in the absence of calcium and speculated that this was possibly a plasma membrane effect. At this time they were unaware of the role of calcium in signal transduction in plants. Several studies of victorin's effect on protoplasts have been conducted, beginning in the mid-1960s. One of the more rigorous such studies was reported by Hawes in 1982, who examined victorin's effect on protoplasts and root cap cells. Protoplasts are sensitive to victorin and
Hawes found differential tissue sensitivity to victorin, with leaf mesophyll cells being at least 10 times more sensitive to victorin than root cap, root epidermal cells or leaf epidermal cells. Hawes also found that even at very high victorin concentrations at high temperatures, several hours were needed for victorin to cause cell death, and she suggested that this is consistent with the hypothesis that the lethal site of action of victorin lies within the cell, not at the plasma membrane. Ullrich and Novacky (1991) used patch-clamp electrophysiology to study the effect of victorin on membrane function and found that Cl⁻ and OH⁻ efflux also occurred from treated cells. Thus, the effect of victorin was not specific to K⁺ channels, but rather a non-specific effect on the plasma membrane. Victorin also did not affect the plasmalemma H⁺-ATPase or lipid structure. They concluded that the effect of victorin on the plasma membrane was most likely secondary and speculated that oxygen radicals and lipid peroxidation might be involved. In 1985, Walton and Earle used protoplasts to show that victorin inhibits protein synthesis and stimulates considerable extracellular polysaccharide synthesis. Another stimulatory effect of victorin was reported in 1986 after victorin was found to stimulate phytoalexin synthesis, but the role of phytoalexins in cell death was apparently secondary, because phytoalexins were not synthesized at higher concentrations of victorin, yet tissue still died (Mayama et al., 1986). Later, victorin was shown to induce phenylalanine ammonia-lyase mRNA (Mayama et al., 1995b). Victorin has also been showed to cause ethylene evolution in treated plants (Shain and Wheeler, 1975). These examples show that not all of victorin’s effects are inhibitory, but that victorin also can stimulate biochemical processes.
Many attempts had been made since the 1950s to purify and identify the structure of victorin, but without success. Wolpert et al. in 1985, identified the structure of victorin as a cyclized pentapeptide as shown in Figure 1-1. They found several forms of the toxin, and the major form was named victorin C and has a molecular weight of 814 Daltons.

![Chemical Structure of Victorin C](image)

**Figure 1.1** Structure of victorin C.

Knowledge of the structure of victorin allowed the construction of $^{125}$I-derivatized victorin, which was biologically active. This $^{125}$I-victorin was used to search for victorin-
binding proteins (VBPs). Leaf slices were treated with $^{125}\text{I}$-victorin and a 100 kDa VBP
was found that bound victorin in leaf slices from susceptible, but not resistant plants
(Wolpert and Macko, 1989). Later, the gene encoding the 100 kDa protein was cloned
and both biochemical and nucleotide sequence analysis indicated the 100 kDa protein is
the pyridoxal-phosphate-containing P-protein of the mitochondrial glycine decarboxylase
complex (Wolpert et al., 1994). This enzyme complex is involved in the
photorespiratory cycle in plants.

Victoria blight only occurs in oats containing the $Pc2$ gene for rust resistance.
Extensive attempts were made to separate crown rust resistance ($Pc2$) from Victoria
blight susceptibility, but neither mutagenic approaches, nor screening millions of
seedlings for naturally-occurring mutants separated rust resistance from Victoria blight
susceptibility (Luke et al., 1960, 1966a). In fact, in these studies, mutants resistant to
victorin were also susceptible to crown rust, while mutants which were less sensitive to
victorin were also less resistant to crown rust. Additional evidence suggesting that the
$Pc2$ allele and the $Vb$ allele are either identical or tightly-linked came from experiments
These results indicated that all tissue culture lines which became victorin-insensitive also
lost the victoria-type crown rust resistance. A recent genetic study of Mayama et al.,
(1995) was also unable to separate $Vb/Pc2$ (Mayama et al., 1995a). Therefore,
identification of the product of the $Vb$ allele, which confers sensitivity to one disease, may
simultaneously identify a crown rust resistance gene of the gene-for-gene type first
described by Flor (1946). Thus, the $C. victoriae$-oat interaction is an especially attractive
model for disease studies because it offers a unique bridge between gene-for-gene and host-selective toxin disease interactions.

REFERENCES


CHAPTER 2

Inhibition of the Glycine Decarboxylase Multienzyme Complex by the Host-selective Toxin Victorin

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ABSTRACT

Victoria blight of oats is caused by the fungus *Cochliobolus victoriae*. This fungus is pathogenic due to its ability to produce the host-selective toxin victorin. We previously identified a 100-kD protein that binds victorin in vivo only in susceptible genotypes and a 15-kD protein that binds victorin in vivo in both susceptible and resistant genotypes. Recently, we determined that the oat 100-kD victorin binding protein is the P protein of the glycine decarboxylase complex (GDC). In this study, we examined the effect of victorin on glycine decarboxylase activity (GDA). Victorin was a potent in vivo inhibitor of GDA. Leaf slices pretreated for 2 hr with victorin displayed an effective concentration for 50% inhibition (EC50) of 81 pM for GDA. Victorin inhibited the glycine-bicarbonate exchange reaction in vitro with an EC50 of 23 μM. We also identified a 15-kD mitochondrial protein that bound victorin in a ligand-specific manner. Based on amino acid sequence analysis, we concluded that the 15-kD mitochondrial protein is the H protein component of the GDC. Thus, victorin specifically binds to two components of the GDC. GDA in resistant tissue treated with 100 μg/mL victorin for 5 hr was inhibited 26%, presumably as a consequence of the interaction of victorin with the H protein. Victorin had no detectable effect on GDA in isolated mitochondria, apparently due to the inability of isolated mitochondria to import victorin. These results suggest that the interaction of victorin with the GDC is central to victorin's mode of action.
INTRODUCTION

We have been investigating the plant disease victoria blight of oats because this host-pathogen interaction has several exploitable characteristics, including the fact that the determinants of specificity and host range are known in this disease interaction. Victoria blight is caused by the fungus *Cochliobolus victoriae* (Meehan and Murphy, 1946), which is pathogenic due to the production of the host-selective toxin victorin. Isolates of *C. victoriae* that produce victorin are pathogenic, whereas isolates that do not are nonpathogenic. Host response to purified victorin parallels host response to the pathogen. Furthermore, host response to the toxin, and therefore the pathogen, is determined by a single gene (*Vb*). Only oat genotypes carrying the dominant *Vb* allele are sensitive to victorin. Consequently, the susceptible and resistant host response can be investigated in the absence of the pathogen, and the analysis of disease specificity can be simplified to an analysis of the interaction between a single fungal metabolite (victorin) with a single dominant plant gene (*Vb*) product.

An additional intriguing characteristic of victoria blight is that identification of the disease susceptibility gene (*Vb*) may lead to the simultaneous identification of a disease resistance gene (*Pc-2*). Victoria blight became a major disease of oat during the 1940s after the release of cultivars carrying the *Pc-2* gene, which gives gene-for-gene-type disease resistance to the crown rust pathogen *Puccinia coronata*. Genotypes carrying the *Pc-2* gene were susceptible to victoria blight, a previously undescribed disease. Various lines of evidence indicate that the *Vb* gene is either closely linked or identical to the *Pc-2* gene (Rines and Luke, 1985). Thus, the *Vb* gene may condition susceptibility to one disease and resistance to another.

We have been studying the mode of action of victorin and attempting to identify the *Vb* gene product. The structure of victorin and procedures for the production of
biologically active $^{125}$I-labeled victorin have been established (Wolpert et al., 1988). Incubation of oat leaf tissue with $^{125}$I-labeled victorin resulted in the identification of a 100-kD victorin binding protein (VBP) that covalently binds radiolabeled victorin in vivo in susceptible but not resistant genotypes (Wolpert and Macko, 1989). A molecular genetic analysis identified the 100-kD VBP as the P protein subunit of the multienzyme glycine decarboxylase complex (GDC) (Wolpert et al., 1994). Four proteins comprise the GDC: P protein, a 100-kD pyridoxal phosphate-containing enzyme; H protein, a 15-kD lipoamide-containing enzyme; T protein, a 45-kD tetrahydrofolate-containing enzyme; and L protein, a 61-kD lipoamide dehydrogenase (Oliver et al., 1990b). The GDC is located in the mitochondrial matrix and along with serine hydroxymethyltransferase catalyzes the conversion of two glycine molecules into serine.

Because victorin binds to the P protein, we decided to investigate whether victorin has any affect on the activity of the multienzyme GDC. This study characterizes the effect of victorin on glycine decarboxylase activity (GDA).

RESULTS

Effect of Victorin on In vivo Glycine Decarboxylation

The activity of the GDC was assayed in vivo by incubating leaf slices with toxin for 2 hr before a 1-hr pulse with carbonyl-labeled $^{14}$C-glycine. The resulting dose response showed that toxin concentrations of ~ 60 ng/mL inhibit glycine decarboxylation greater than 90% in susceptible genotypes as shown in Figure 2.1A. Victorin had an effective concentration for 50% inhibition (EC$_{50}$) of GDA of 0.07 ng/mL (81 pM) in leaf slices exposed to toxin for 2 hr. Increasing the amount of time leaf slices were incubated with toxin increased the inhibition of GDA. For example, GDA in leaf slices incubated for 2
hr in buffer followed by 5 hr with toxin and a 1-hr pulse with \textsuperscript{14}C-glycine was inhibited 14\% by 0.005 ng/mL victorin. GDA was inhibited 61\% in leaf slices treated with 0.01 ng/mL of toxin under the same conditions. Thus, following a 5-hr exposure to toxin, the EC\textsubscript{50} of victorin on GDA was below 0.01 ng/mL (12 pM).

A comparison with previously described inhibitors of glycine decarboxylation indicated that victorin is as inhibitory to GDA at nanomolar concentrations as the known glycine decarboxylation inhibitors cysteine, serine or glycine hydroxamate are at millimolar concentrations. As shown in Figure 2.1B, 1.2 nM victorin (1 ng/mL) inhibits GDA to approximately the same extent as 100,000 fold higher concentrations of the other GDA inhibitors.

Because victorin is a potent phytotoxin, treatment of sensitive genotypes with victorin ultimately leads to cell death. Presumably, as a consequence of victorin's interaction with its site of action, many cellular activities are indirectly perturbed before cell death occurs. Effects of victorin on sensitive tissues include electrolyte leakage, membrane depolarization, extracellular polysaccharide production, increased respiration, and inhibition of dark CO\textsubscript{2} fixation (Walton and Earle, 1985; Wolpert et al., 1988; Ullrich and Novacky, 1991). To investigate the possibility that the effect of victorin on GDA in vivo could be the result of general cellular disruption, as opposed to a specific effect of victorin directly on GDA, we compared the in vivo effect of victorin on GDA with light-dependent CO\textsubscript{2} fixation, another organelar process. We found that the in vivo effect of victorin on light-dependent CO\textsubscript{2} fixation was approximately 350 times less inhibitory than on GDA (Figure 2.2). The EC\textsubscript{50} for the inhibition of light-dependent CO\textsubscript{2} fixation was 66 ng/mL (81 nM) as compared to 0.19 ng/mL (0.23 nM) for GDA after exposure to toxin for identical times as shown in Figure 2.2.
**In vitro Effects of Victorin on GDA**

A mitochondrial matrix extract was used for glycine-dependent bicarbonate exchange reactions. Glycine-bicarbonate exchange activity was examined because the reaction is only dependent upon the P and H proteins of the GDC and the T and L proteins are not required (Sarojini and Oliver, 1983). Thus, the reaction can be performed aerobically unlike glycine decarboxylation reactions, which require an anaerobic environment to avoid oxidation of tetrahydrofolate, a required cofactor. Extracts from susceptible plants were incubated for 75 min with or without toxin after which the glycine exchange reaction was initiated and the reaction allowed to continue for 15 min as illustrated in Figure 2.3A. The EC₅₀ of victorin for inhibition of glycine exchange activity was 19 μg/mL (23 μM). Victorin inhibited the glycine-bicarbonate exchange reaction in samples from both susceptible and resistant genotypes. This is consistent with in vitro binding studies that demonstrated victorin binding by P protein in extracts from both genotypes (Wolpert and Macko, 1989).

During binding studies with victorin, we observed that in vitro binding of ¹²⁵I-labeled victorin by the P protein was stimulated approximately 23% by higher salt concentrations as illustrated in Figure 2.3B. Because the P protein bound victorin more effectively in high-salt conditions, we examined the effect of adding salt to the exchange reactions. The presence of 25 mM KCl resulted in a 96% increase in exchange activity. Victorin was a more effective inhibitor of exchange activity in the presence of KCl (Figure 2.3A). Thus, conditions which increased binding of toxin to the P protein resulted in increased inhibition of the exchange reaction by victorin.
Figure 2.1. In vivo inhibition of GDA.

(A) Leaf slices were treated for 2 hr with the indicated victorin concentration, followed by a 1-hr incubation with 500 nCi of $^{14}$C-glycine. The percent inhibition of GDA relative to nontreated controls is shown. Data points represent the means ± SE of four experiments with three replications per treatment.

(B) Leaf slices were treated for 2 hr with the indicated GDC inhibitors. Data points represent the means ± SE of three experiments with three replications per treatment. GH, glycine hydroxamate.
Victorin Binding to the H Protein

Previous work revealed that a 15-kD protein also bound $^{125}$I victorin in vivo and in vitro (Wolpert and Macko, 1989). However, unlike the binding to the 100-kD VBP that occurs in vivo only in susceptible genotypes, binding to the 15-kD VBP occurs in both susceptible and resistant genotypes. During purification of the P protein from mitochondria, we observed that a 15-kD VBP was also enriched in the mitochondrial fraction. We purified this protein from isolated mitochondria and determined that it bound $^{125}$I victorin in a ligand-specific manner as shown in Figure 2.4A. The N-terminal amino acid sequence identified the first 30 amino acid residues of the 15-kD VBP purified from mitochondria (Figure 2.4B). The amino acid sequence showed 77% amino acid identity with the first 30 amino acid residues of the mature H protein deduced from a cDNA isolated from pea and 73% with the deduced mature form of the Arabidopsis H protein (Figure 2.4B) (Kim and Oliver, 1990; Srininivasan and Oliver, 1992). These results indicated that victorin binds in a ligand-specific manner to the H protein component of the GDC from oats. Thus, victorin binds to two different components of the GDC in a ligand-specific manner. Our current hypothesis is that the 15-kD protein labeled in vivo is the same protein that is labeled in vitro, the H protein.

Glycine Decarboxylase in Roots and Etiolated Tissue

Victorin is toxic to roots (Hawes, 1983), a non-photorespiratory tissue. We have found that etiolated blades treated with victorin in the dark are sensitive to toxin. Because non-
green tissues are sensitive to victorin, we sought to confirm that oats, like other plants, have the GDC present in non-green tissue. The GDC is known to be strongly light induced in plants, and green tissue has approximately 10-fold more GDC than etiolated tissue (Walker and Oliver, 1986). Mitochondria were isolated from roots, etiolated blades, and etiolated blades that had been exposed to light for 14 hr before harvesting. Mitochondrial protein was separated using SDS-PAGE and analyzed by protein blotting. P protein was detected with anti-100-kD VBP antibody in both roots and etiolated blades as seen in Figure 2.5. Thus, as in other plants, the oat GDC is present in non-green tissues and is light-inducible (lane 3).

**Treatment of Isolated Mitochondria with Victorin**

The effect of victorin on GDA in intact mitochondria isolated from susceptible and resistant oat blades was evaluated. Victorin appeared to have no effect on GDA in isolated mitochondria (data not shown). GDA was not inhibited in isolated mitochondria pretreated with 100 μg/mL of victorin for 1 hr before the addition of 14C-glycine. Failure of isolated mitochondria to import victorin might explain the lack of a detectable effect of toxin on isolated mitochondria. To evaluate toxin uptake, we examined whether 125I-victorin would label P protein in intact, isolated mitochondria. Because P protein is a matrix protein it will become freely soluble if mitochondria lose their integrity. Also, it has been established that soluble P protein will bind victorin (Wolpert and Macko, 1989). Therefore, it was necessary to determine whether any labeled P protein detected in toxin-treated mitochondrial preparations was within the matrix of intact mitochondria or in the soluble fraction as a consequence of mitochondrial lysis.
Figure 2.2. Comparison of in vivo victorin inhibition of light-dependent CO$_2$ fixation and GDA.

Leaf slices were treated with toxin for 90 min before a 20-min pulse with $^{14}$C-glycine (■) or NaH$^{14}$CO$_3$ (□). Results are shown as the percentage of inhibition of activity relative to non-treated controls. Light-dependent CO$_2$ fixation data points represent the mean ± SE of four experiments with three replications each, and the GDA data points are the mean ± SE of three experiments with three replications each.
Figure 2.3. In vitro inhibition of glycine-bicarbonate exchange by victorin.

(A) Samples containing mitochondrial extracts, with 25 mM KCl (■) or without KCl (□), were incubated with victorin for 75 min after which a 15-min reaction was initiated with NaH\(^{14}\)CO\(_3\). Data points represent the means ± SE of seven to nine experiments with two replications per treatment and are expressed as the percentage of inhibition of glycine-bicarbonate exchange or nontreated controls.

(B) Shown is an autoradiograph of a SDS-polyacrylamide gel of freeze-thawed mitochondria (6 μg of protein per lane), plus (lane 2) or minus (lane 1) 50 mM KCl, that was incubated for 45 min with 0.3 μCi/mL 125I-victorin. Numbers at left indicate the location of protein size markers.
Figure 2.4. Analysis of the 15-kD oat victorin binding protein.

(A) This is an autoradiograph of a polyacrylamide gel following electrophoresis of purified 15-kD VBP. Prior to electrophoresis samples were incubated in the presence of 3 μCi/mL of 125I-labeled victorin for 1 hr. Binding assays were conducted in the presence of 0, 0.1, 1, 10, and 100 μg/mL of unlabeled victorin. The marker at left indicates the H protein.

(B) N-terminal amino acid sequence of the mature 15-kD VBP isolated from susceptible oat leaf tissue (A.s.); N-terminal amino acid sequence of mature H protein deduced from cDNAs cloned from pea (P.s.) and Arabidopsis (A.t.). The numbers above the amino acid sequences indicate amino acid positions.
Figure 2.5. Protein gel blot analysis of 100-kD VBP from non-green oat tissues.

Mitochondria were isolated from roots, etiolated blades, and etiolated blades transferred to light for 14 hr. Lane 1 contains 5 µg of root mitochondrial protein; lane 2, 0.5 µg of mitochondrial protein from etiolated blades; lane 3, 0.5 µg of mitochondrial protein from etiolated blades exposed to light for 14 hr before mitochondrial isolation. The position of the 100-kD VBP is indicated at left.
Mitochondria were incubated with $^{125}$I-labeled victorin for 40 min and then centrifuged. The mitochondrial supernatant and pellet were separated and analyzed. We compared the distribution of labeled and unlabeled P protein between the mitochondrial pellets and supernatants. Labeled P protein was quantitated by densitometric scans of autoradiographs of SDS-polyacrylamide gels. Total P protein was estimated by densitometric scans of the 100-kD protein band detected in Brilliant Blue G stained SDS-polyacrylamide gels. To ensure that the 100-kD protein scanned was the P protein, we separated mitochondrial proteins by 2-dimensional gel electrophoresis. Only one protein that migrated with an apparent mass of 100 kD was detected in stained gels. This protein was immunoreactive with the P protein antibody (D.A. Navarre and T.J. Wolpert, unpublished results). Therefore, the 100-kD band detectable on Brilliant Blue G stained SDS-polyacrylamide gels is primarily the P protein. This finding is consistent with the observation that the GDC is the most abundant mitochondrial enzyme in green tissues (Walker and Oliver, 1986). Quantitation of P protein by direct staining was considered more accurate than protein blotting because transfer of P protein from SDS-polyacrylamide gels to membranes is not quantitative.

The majority of labeled P protein (100-kD VBP) was found in the supernatant as shown by the autoradiograph in Figure 2.6B, whereas the pellet contained only a small amount of labeled P protein. However, as expected, the majority of the P protein was in the mitochondrial pellet and not the mitochondrial supernatant as shown in Figure 2.6A. Comparison of the densitometric analysis of the autoradiographs and Brilliant Blue G stained polyacrylamide gels revealed that 76% of the P protein but only 7% of the total labeled P protein was detected in the mitochondrial pellet, whereas 93% of the labeled P protein was detected in the supernatant (Table 1). Thus, when isolated mitochondria are treated with victorin, 93% of the labeled P protein is found in the mitochondrial supernatant, whereas the majority of the P protein is in the intact, pelletable mitochondria.
We sought to determine whether the small amount of labeled P protein in the pellet was due to victorin that had been imported into intact mitochondria or whether this binding was attributable to externally labeled P protein adsorbed to the mitochondrial surface and/or a small proportion of membrane-damaged mitochondria in the pellet. Protease treatments were unsuccessful in determining whether the small amount of labeled P protein associated with the mitochondrial pellet was compartmentalized within mitochondria, because protease concentrations sufficient to digest labeled P protein known to be externally associated with the mitochondria resulted in the loss of mitochondrial integrity (data not shown). Thus, as an alternative approach, mitochondria were frozen and thawed three times and then treated with 125I-labeled victorin (Figure 2.6B) as described above. In this instance, 74% of the P protein was in the supernatant and 96% of the labeled P protein was also in the supernatant (Table 1). Even though the mitochondria had been subjected to three-freeze-thaw cycles, 4% of the labeled P protein still remained in the pellet (Figure 2.6B). The most plausible interpretation of these results is that victorin is not imported by intact isolated mitochondria and that the small amount of label found in the mitochondrial pellets is due to a combination of permeabilized mitochondria and/or decompartmentalized P protein associated with mitochondrial membranes. Victorin import into isolated mitochondria was not stimulated in the presence of ADP or ATP, or with various mitochondrial substrates, including glycine, succinate, and malate (D. A. Navarre and T. J. Wolpert, unpublished results).
Figure 2.6. Analysis of $^{125}$I-victorin binding by isolated mitochondria.

Isolated mitochondria (50 μg of protein) that were either intact or that had been subjected to three freeze-thaw cycles were labeled with $^{125}$I-victorin for 40 min. Samples were then separated into supernatant and pellet fractions and resolved by SDS-PAGE.

(A) Brilliant Blue G stained gel. Lane 1 contains the pellet fraction of intact mitochondria; lane 2, pellet from freeze-thawed mitochondria; lane 3, supernatant fraction from the sample with intact mitochondria; lane 4, supernatant from the sample with freeze-thawed mitochondria.

(B) Autoradiograph of the same gels exposed for 6 hr. Numbers at left indicate the 100-kD protein.
Table 2.1 $^{125}$I-victorin labeling analysis of isolated oat mitochondria.

<table>
<thead>
<tr>
<th>Mitochondrial Fraction</th>
<th>% Total 100-kD protein</th>
<th>% Total $^{125}$I-victorin binding by P protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact pellet</td>
<td>76</td>
<td>7</td>
</tr>
<tr>
<td>Supernatant from intact pellet</td>
<td>24</td>
<td>93</td>
</tr>
<tr>
<td>Pellet from freeze-thawed sample</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>Supernatant from freeze-thawed sample</td>
<td>74</td>
<td>96</td>
</tr>
</tbody>
</table>

Isolated mitochondria were either intact or freeze-thawed three times and then labeled with $^{125}$I-victorin for 40 min. Samples were separated into supernatant and pellet fractions and resolved by SDS-PAGE. The relative amount of 100-kD protein in the supernatant versus pellet was determined by scanning the SDS-polyacrylamide gels. The relative amount of radiolabeled P protein in the supernatant versus pellet fraction was determined by scanning an autoradiograph of SDS-polyacrylamide gels. Data represent the average of two replicates.
Effect of Victorin on Glycine Decarboxylation in Resistant Genotypes

Because the 15-kD VBP labeled in vitro was identified as the GDC H protein, it is possible that the 15-kD VBP labeled in vivo in both susceptible and resistant genotypes is the H protein. If the H protein is the protein labeled in vivo in resistant genotypes, then victorin might also have an effect on GDA in resistant genotypes despite the fact that the P protein is not labeled. We measured the effect of high victorin concentrations on GDA in leaf slices from resistant genotypes. Leaf slices from the resistant genotype X424 were incubated with 100 µg/mL of toxin for 5 hr before measuring GDA. An inhibitory effect was observed that was far less pronounced than that seen in the susceptible line X469 and required much higher toxin concentrations. In one representative experiment (10 replications per treatment), in which samples received 100 µg/mL toxin, GDA was inhibited 26.5% relative to control samples without toxin (SE of 1.5%, P value of 9 x 10^-11). Although crude preparations of C. victoriae extracts have been shown to have effects on resistant plants, no effect has been previously observed with purified victorin concentrations as high as 100 µg/mL (Scheffer and Livingston, 1984). This effect is most likely due to the interaction of victorin with the H protein subunit of the GDC. No effect on GDA was observed with the toxin concentrations used to generate the dose-response for sensitive plants (Figure 2.1A). Leaves from resistant plants treated with 100 µg/mL victorin concentrations displayed no visible symptoms.
DISCUSSION

In this study, victorin was found to be a potent inhibitor of GDA in vivo, with an EC$_{50}$ of 0.07 ng/mL (81 pM) (Figure 2.1A) in leaf slices pretreated for 2 hr with victorin. GDA inhibition by as little as 5 pg/mL victorin was observed after a 5-hr toxin preincubation. We evaluated the possibility that inhibition of GDA might be an indirect effect of victorin. Because glycine decarboxylation occurs in an organelle, we compared GDA to another organellar process, light-dependent CO$_2$ fixation, based on the assumption that an indirect perturbation of organellar functions would be similar for all organelles. Light-dependent CO$_2$ fixation was about 350 times less sensitive to toxin (Figure 2.2) and was thus presumed to be an indirect effect and indicative of generalized cellular disruption. Additional evidence that victorin directly inhibited the GDC was shown by the in vitro inhibition of glycine-bicarbonate exchange (EC$_{50}$ of 23 μM). Furthermore, victorin binding by the P protein was enhanced by high-salt concentrations as was the inhibition of glycine-bicarbonate exchange activity by victorin. To our knowledge, no effect of victorin on cell-free preparations has been reported (Scheffer and Livingston, 1984).

Victorin inhibited GDA at lower concentrations in vivo than in vitro. This may be due in part to the fact that the glycine-bicarbonate exchange reaction was used for in vitro assays and the glycine decarboxylation reaction for in vivo assays. However, a more likely explanation may be that the inhibition of GDA in vivo might cause perturbations upstream in the photorespiratory pathway and disrupt the cooperative interactions of chloroplasts, peroxisomes, and mitochondria that occur during photorespiration. If glycine concentrations rise due to the inhibition of GDA, the plant may shunt glycine and glycine precursors into different metabolic pathways. Inhibition of GDA may also have deleterious effects on mitochondria, such as reduced ability to import exogenous
substrates, including glycine. Victorin may also be less inhibitory in vitro due to differences in the structure of the GDC in vivo versus in vitro. In vivo, the GDC is comprised of 42 subunits and has a molecular mass of greater than 1.3-MD (Oliver et al., 1990b). As pointed out by Neuburger et al. (1991), the recognition processes of the intact complex in the mitochondrial matrix, where it exists in a concentrated state (130 mg/mL in pea), are different from the dissociated form of the complex in vitro. Thus, the interaction of victorin with the complex in vivo versus in vitro is possibly quite different. This is consistent with our previous finding that in vivo the P protein only binds victorin in sensitive genotypes, whereas in vitro this specificity is lost and P protein from both susceptible and resistant plants binds toxin. Another explanation for the observation that victorin inhibited GDA at lower concentrations in vivo than in vitro might be that victorin is modified to a more toxic form in vivo. Finally, the fact that picomolar amounts of victorin inhibited GDA in vivo may indicate that victorin is concentrated by the plant--an event that will not occur when GDA is assayed with enzyme extracts.

**Victorin Labels Mitochondria In vivo but not In vitro**

Given the effect of victorin on GDA in leaf slices and on mitochondrial matrix extracts, we were surprised to find that victorin had no discernible effect on GDA in isolated mitochondria. This is consistent with earlier findings that indicated that victorin did not directly affect respiratory activity in isolated mitochondria, despite the respiratory burst observed with treated, intact tissue (Wheeler and Hanchey, 1966). We examined victorin binding by isolated mitochondria to determine if toxin uptake could explain the discrepancy between the inhibition of GDA both in vivo and in vitro but the lack of effect on GDA in isolated mitochondria. Victorin did not appear to be imported by isolated mitochondria (Figure 2.6). A small amount of 125I-victorin binding (7% of total binding) was associated with intact mitochondria (Table 1); however, we believe this binding is
due to either damaged mitochondria and/or liberated P protein associated with the outside of mitochondrial membranes.

Victorin import by mitochondria in vivo is indicated by the observations that (1) the 100-kD VBP is labeled in vivo and is the P protein component of a mitochondrial enzyme complex (Wolpert et al., 1994); (2) the 15-kD VBP labeled in vivo may be the H protein component of the same enzyme complex; and (3) in vivo labeling with biotinylated victorin shows differential labeling of intact mitochondria from susceptible and resistant genotypes (H. Israel, T. J. Wolpert, and V. Macko, unpublished data). Possible mechanisms for victorin import into mitochondria might include the requirement of a carrier protein, the requirement for another cellular component, the need for a particular mitochondrial substrate or cofactor, or for victorin modification prior to mitochondrial import. In considering the latter possibility, it is interesting that the glyoxylic acid residue covalently bound to the dichloroleucine residue of victorin C is important for its activity (Wolpert et al., 1988). Glyoxylic acid is the immediate precursor to photorespiratory glycine. Furthermore, Kono et al. (1986) identified a form of victorin (victorin M) in which the glyoxylic acid residue was replaced with a glycine residue; this suggests that victorin M may be deaminated to form victorin C.

If the mitochondrion is the site of action for victorin, then genotypical specificity could occur at the point of victorin import into mitochondria or the cell. However, if the 15-kD protein labeled in vivo in susceptible and resistant genotypes is the same as the 15-kD protein (H protein) labeled in vitro, then victorin is apparently imported into the mitochondria of both susceptible and resistant genotypes. Supporting this is the fact that high concentrations of victorin inhibited GDA in resistant plants. These data suggest that differential mitochondrial uptake of victorin is not the basis for the genotype-specific effects of victorin or the explanation for genotype-specific binding of victorin to the 100-kD P protein from susceptible genotypes.
Interactions of Victorin and the GDC.

The interaction of victorin with GDC may be sufficient to cause cell death. However, phytotoxicity could result as a consequence of three possibilities. First, victorin is selectively metabolized by resistant genotypes and rendered nontoxic. Conceivably, the GDC could be responsible for such detoxification. It has been suggested that victorin is degraded by resistant plants (Wheeler, 1969). In a second possibility, victorin is metabolized by this enzyme complex in susceptible genotypes and a resulting victorin metabolite causes cell death. Supporting this possibility is the existence of victorin forms with glyoxylic acid or glycine residues—both photorespiratory substrates. Furthermore, that victorin binds two different components of GDC may be indicative of processing by the complex. The H protein with its flexible lipoamide arm interacts with the active sites of the P, T and L proteins. Thus, toxin might also interact with T and L proteins in addition to P protein. We have found that victorin binds to two components of GDC. The other two proteins of the GDC have molecular masses in pea of 45 kD (T protein) and 61 kD (L protein) (Bourguignon et al., 1988)—intriguingly close in size to the 45- and 65-kD VBPs detected by Akimitsu et al., (1992) using anti-victorin antibody. An explanation for the detection of different victorin binding proteins by the different approaches may be due to the nature of victorin detection used and metabolism of victorin by the plant. Our approach can detect proteins that bind victorin only if the 125I-group remains on the part of victorin bound, whereas the anti-victorin antibody could detect a different part of the victorin molecule.

A third possibility is that cell death is a direct consequence of the inhibition of the GDC. GDC photorespiratory mutants have been shown to be lethal (Somerville and Ogren, 1982). Thus, nonfunctional GDC can be lethal in plants. Victorin is toxic to roots (Hawes, 1983), a non-photorespiratory tissue and etiolated leaf blades. Because non-photorespiratory tissues are sensitive to victorin, we must consider the possibility that the
GDC has non-photorespiratory biochemical functions that lead to cell death if disrupted. The GDC is found in roots and etiolated tissues of oat and other plants and in C4 plants, which suggests non-photorespiratory roles in plants. Furthermore, the GDC is found in a wide range of organisms from bacteria to humans and, thus, obviously has functions unrelated to photorespiration. Defective GDC is the cause of hyperglycinemia, a lethal, incurable disease in humans (Kume et al., 1988). In *Escherichia coli*, 15% of all carbon atoms assimilated from glucose are thought to enter the glycine-serine pathway, and this pathway is also the major source of one-carbon units in the cell (Wilson et al., 1993).

While photorespiration may not be necessary for victorin-mediated cell death, it might be expected that under photorespiratory conditions green tissue might be less tolerant to the inhibition of GDA and consequently more sensitive to victorin. Green tissue may be more sensitive to toxin because it has approximately 10-fold more GDC than etiolated tissue (Walker and Oliver, 1986). Interestingly, leaf protoplasts have been reported to be 10 times more sensitive to victorin than root tissue (Hawes, 1983).

The interaction of victorin with the GDC could conceivably have many consequences. Inhibiting the complex in photorespiratory conditions could result in a rapid inhibition of photosynthesis, which in turn will have its own cascade of effects. Furthermore, inhibiting the GDC may prevent the T protein from replenishing the mitochondrial pool of tetrahydrofolate. In addition, if toxin interacts with or inhibits the L protein, then pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase may also be affected because the L protein is also a constituent of these two complexes (Bourguignon et al., 1992). Interestingly, of the four GDC proteins, the L protein is expressed the most in non-green tissue (Bourguignon et al., 1992).

Thus, several lines of evidence suggest that victorin's interaction with the multienzyme GDC is pivotal: (1) victorin is a potent inhibitor of GDA in vivo; (2) victorin inhibits GDC glycine-bicarbonate exchange in vitro; (3) in addition to binding to the P protein, 125I-victorin binds to a 15-kD protein, which we have identified as the H
protein of the GDC, and therefore, victorin binds in a ligand-specific manner to two components of the GDC in vitro; (4) victorin is bound in vivo by the P protein in susceptible but not resistant cultivars; (5) victorin inhibits GDA in resistant tissue; (6) all known forms of victorin have structural components (glyoxylic acid or glycine) that are key substrates of the photorespiratory pathway.

Our current hypothesis is that the mitochondrion is the primary site of action of victorin, specifically at the GDC, and that a GDC component may be the product of the \( V_b \) gene. However, GDC or a component of the GDC could also be the site of action without being the product of the \( V_b \) gene. In this scenario, the \( V_b \) gene product would presumably directly or indirectly mediate the interaction of victorin with a GDC protein. Alternatively, a GDC protein could be the product of the \( V_b \) gene without being the site of action. In this instance, the GDC component might modify toxin in susceptible genotypes only, and the resulting metabolite could act elsewhere.
METHODS

Plant Material

Oat seedlings were grown in a growth chamber for 5-7 days under a 16-hr photoperiod at 24°C. The resistant oat line X424 and susceptible lines X469 and Park were used. Etiolated plants were grown for 1 week at 24°C in a growth chamber and fertilized with nutrient solution (Moore, 1981). Etiolated plants receiving light treatment were exposed to light for 14 hr on day 7 before being harvested. Roots were excised from seedlings grown for 6-8 days on cheesecloth over an aerated nutrient solution (Moore, 1981).

Isolation of mitochondria

Leaves from 5- to 7-day-old seedlings were homogenized with a cold mortar and pestle in cold isolation buffer (400 mM mannitol, 0.3% PVP, 1 mM EDTA, 0.1% defatted BSA, 30 mM 2-[N-morpholino]propane sulfonic acid [Mops], pH 7.5). The homogenate was filtered through two layers of cheesecloth and centrifuged at 800 g for 5 min. The supernatant was collected and centrifuged at 12000 g for 10 min. The pellet was gently resuspended in isolation buffer and fractionated on Percoll gradients essentially as described by Douce et al. (1987). The purified mitochondria were collected and rinsed twice with suspension buffer (300 mM mannitol, 10 mM Mops, 1 mM EDTA, 2 mM MgCl₂, pH 7.2).

Glycine Decarboxylation assays

Leaf slices prepared as described by Wolpert et al. (1988) were used for the in vivo assay of glycine decarboxylation. Ten leaf slices were aliquoted into serum vials containing 500 μL of 10 mM Mops, pH 7.2, and allowed to equilibrate for 1 hr with gentle shaking inside a 30°C water bath incubator. Victorin was added, and the vials were incubated for 2 hr, which was followed by the addition of 500 nCi of 1-14C-glycine (51 mCi/m mole;
ICN, Costa Mesa, CA). Glycine decarboxylation was terminated after 1 hr with 100 μL of 50% (v/v) acetic acid. Liberated CO₂ was trapped on filter paper impregnated with 150 μL of 2 N NaOH. Filters containing trapped CO₂ were placed in 4 mL of CytoScint ES (ICN) and counted in a liquid scintillation spectrometer. Inhibitors of glycine decarboxylation were assayed in the same manner except 50 mM Mops, pH 7.2 was used as the buffer.

In vitro glycine decarboxylase activity was measured by the glycine-bicarbonate exchange reaction (Sarojini and Oliver, 1983). Percoll-purified mitochondria from 50-100 g of tissue were rinsed with wash buffer (400 mM mannitol, 5 mM Tris, 5 mM Mops, 1 mM EDTA, 2 mM DTT, pH 7.5), and suspended in 1.5 mL of extraction buffer (5 mM Tris, 5 mM Mops, 1 mM EDTA, 2 mM DTT, pH 7.5); the matrix protein was released with three or four freeze-thaw cycles. After each cycle, the samples were centrifuged for 3 min at 12,000 rpm in a Sorvall (Norwalk, CT) MC 12V microcentrifuge. The supernatant was saved and the pellet reextracted. Supernatants were pooled and centrifuged at 100,000 g for 1 hr to pellet mitochondrial membranes. The resulting mitochondrial matrix extract was used in the glycine-bicarbonate exchange reactions. Assays were conducted in a 100 μL reaction volume containing 40 μg of protein, 50 μM pyridoxal phosphate, 2 mM DTT, 20 mM glycine, and 3 μCi of NaH¹⁴CO₃ (3 mCi/mmole) in extraction buffer. Samples were incubated with victorin for 75 min at 30°C after which glycine, pyridoxal phosphate and NaH¹⁴CO₃ were added. The reaction was stopped with 50% (v/v) acetic acid after 15 min. Protein concentrations were determined with the Bio-Rad protein assay with BSA as the standard (Bradford, 1976).

**Light-dependent CO₂ fixation assay**

For the comparison of the effect of victorin on light-dependent CO₂ fixation versus glycine decarboxylation, 10 leaf slices were incubated inside a covered, shaking water
bath for 1 hr in 500 µL of 10 mM Mops, pH 7.2, followed by a 90-min toxin treatment. Samples were then incubated for 20 min with either 500 nCi of $^{14}$C-glycine (51 mCi/mmol) or 1 μCi of $^{14}$C-bicarbonate (1 mCi/mmol). In the CO$_2$ fixation assays, vials were transferred to high light (11,000 lux) for the duration of the reaction and 5 min prior to the addition of bicarbonate. Reactions were stopped with 100 µL of 50% (v/v) acetic acid; for the CO$_2$ fixation assay, the remaining acid-stable radioactivity was counted. Light-dependent CO$_2$ fixation reactions were conducted in scintillation vials under high light intensity, and a water bath was used to minimize transfer of radiant heat.

**Labeling of mitochondria with $^{125}$I-victorin**

Isolated mitochondria were used immediately for $^{125}$I-victorin binding studies. Radiolabeled victorin was prepared as previously described (Wolpert and Macko, 1989). Mitochondria (50 μg of protein) that were either intact or ruptured with three freeze-thaw cycles were incubated for 40 min in suspension buffer at 25°C in a 30 µL reaction containing 6 mM DTT and 6 μCi/mL $^{125}$I-victorin. Samples were then centrifuged at 4°C for 5 min at 8000 rpm in a microcentrifuge. The supernatant was removed, and the pellet was washed twice with 1.5 mL of cold suspension buffer and pelleted as described above. Samples were mixed with 10 µL of gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 10% [w/v] sucrose) and then separated by electrophoresis in 14% polyacrylamide gels containing SDS. Radiolabeled proteins were analyzed by autoradiography, and proteins were stained with colloidal Brilliant Blue G reagent (Sigma). Scans of gels and autoradiographs were generated with a BioPhotronics Gelprint 2000i imaging system and bands were quantified with GPTools v3.0 software (BioPhotronics, Ann Arbor, MI).
Purification and identification of the 15-kD victorin binding protein

Mitochondrial matrix protein was prepared as described above. Matrix protein was adjusted to 100 mM KCl by the addition of solid KCl and applied to a hollow-fiber, centrifugal concentrator (RCF-ConFilt, Bio-Molecular Dynamics, Beaverton, OR). The majority of matrix proteins were retained by the concentrator, but the 15-kD victorin binding protein (VBP) was not. The material that had passed through the concentrator was mixed with three volumes of 20 mM Tris, HCl, 1 mM EDTA, 2 mM DTT, pH 8.0 (buffer A), and applied directly to a Mono-Q anion exchange column (Pharmacia), which had been equilibrated in buffer A. The protein was then eluted with a 120-min linear gradient of 0-1.0 M NaCl in buffer A. Fractions were analyzed by SDS-PAGE, and fractions containing the 15-kD protein were pooled. In vitro binding was assessed by the addition of 3 μCi/mL of \[^{125}\text{I-victorin}\] to protein fractions and incubating the samples for 1 hr at 25°C. The reaction was terminated by mixing samples with gel loading buffer. Samples were then subjected to electrophoresis on 14% polyacrylamide gels containing SDS. Radiolabeled proteins were analyzed by autoradiography. The N-terminal amino acid sequence was determined on an Applied Biosystems (Foster City, CA), Model 475A gasphase protein sequencer operated by the Central Service Lab of the Center for Gene Research and Biotechnology (Oregon State University).

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CHAPTER 3

Effects of Light and CO₂ on Victorin Symptom Development

INTRODUCTION

The fungus *Cochliobolus victoriae* causes victoria blight of oats (Meehan and Murphy, 1946). *C. victoriae* produces the pentapeptide toxin victorin, which is the primary determinant of both pathogenicity and host-range. *C. victoriae* strains which do not make victorin are non-pathogenic and only oat lines susceptible to the fungus are sensitive to the toxin. Victorin only affects oat lines carrying the dominant *Vb* allele. No other tested organisms, including plants, have been found to be sensitive to victorin.

Victorin is readily taken up by excised leaves and the effects of victorin on symptom development are seen at concentrations below 10 nanomolar. Leaves treated with 100 ng/ml of victorin die within 24 hours and the major symptom is severe wilting. The basis for the specificity and mode of action of victorin has been unknown, but recent evidence implicates the mitochondrial enzyme complex, glycine decarboxylase (Navarre and Wolpert, 1995). Radiolabeled victorin was found to bind two (P- and H-proteins) of the four protein components that comprise the glycine decarboxylase complex (GDC) (Wolpert et. al., 1994 and Navarre and Wolpert, 1995). Fifty percent inhibition of GDC activity in leaf slices treated for 2 hours with victorin occurs at 81 pM (Navarre and Wolpert, 1995). This suggests a component of the GDC complex might be the product of
the Vb gene and/or that inhibition of this complex may be central to the mode of action of victorin. The GDC is composed of four different proteins: P protein, a 100-kD pyridoxal phosphate-containing enzyme; H protein, a 15-kD lipoamide-containing enzyme; T protein, a 45-kD tetrahydrofolate-containing enzyme; and L protein, a 61-kD lipoamide dehydrogenase (Oliver et al., 1990b). The GDC is important in reclaiming carbon lost due to photorespiration. Non-functional GDC is apparently lethal because barley and Arabidopsis mutants compromised in GDC activity die under photorespiratory conditions (Somerville and Ogren, 1982; Blackwell et al., 1990; Artus et al., 1994). Thus, the GDC represents a reasonable site of action for victorin.

Victorin is toxic under non-photorespiratory conditions, killing leaves incubated in the dark, and is also toxic to roots. Thus, victorin may also have effects separate from the inhibition of GDC, or GDC may be important in non-photorespiratory conditions. Previously, Hawes (1983) found that oats have differential tissue sensitivity to victorin, and that root cells are approximately 10 times less sensitive to victorin than leaf mesophyll cells, which correlates well with the apparent level of the GDC in non-green tissues (cf. Walker and Oliver, 1986, Navarre and Wolpert, 1995). If the inhibition of GDC by victorin causes cell death or if plants have a greater need for glycine decarboxylation under photorespiratory conditions, then one would expect to see an alteration in the plant response to victorin in photorespiratory versus non-photorespiratory. In this paper, we report on the effects of light or CO₂ manipulation on the development of the symptoms of victorin development.
METHODS

Plant Material

Oat seedlings were grown in a growth chamber for 6-8 days using a 16-hr photoperiod at 24°C. The resistant oat line X424 and susceptible line X469 were used. In addition to daily watering, plants were watered twice with a nutrient solution (Moore, 1981).

Gas Treatments

Six to 8 day old leaves were detached and pretreated for 1 hour with air supplemented with either 1% or 5% CO₂. After one hour leaves were incubated in either 1 ng/ml, 10 ng/ml, or 100 ng/ml victorin in 10 mM MOPS, pH 6.5. Gas concentrations were controlled by a Matheson multiple DNYA-Blender, model 8219 Gas Controller. Leaves were incubated in either darkness or light for 24 or 48 hours. Concurrently, otherwise identically treated leaves were incubated in normal air.

RESULTS

Effect of Light or Darkness

Victorin-treated leaves were examined for any visible difference in response to victorin when incubated in light versus darkness. Leaves were detached from 6-8 day old seedlings and incubated in either continual light or darkness for one to three days. Symptoms appeared more severe when leaves were incubated in the light (Figure 3.1). Leaves treated with 100 ng/ml in the light were shriveled and desiccated by 24 hours after treatment, whereas dark-incubated leaves were showing only the first signs of wilting,
and the major symptom was loss of chlorophyll relative to untreated leaves (Figure 3.1A). Leaves treated in the light with 10 ng/ml of victorin for 24 hr were turgid and the primary symptom was loss of chlorophyll (Figure 3.1B), whereas dark incubated leaves showed no symptoms. After 48 hours, leaves incubated with 10 ng/ml victorin in the light showed extensive yellowing and were moderately shriveled (Figure 3.1D), while dark-incubated blades remained turgid and were just beginning to yellow. A differential response by leaves to victorin in the light or dark was particularly evident when leaves were treated with 1 ng/ml (Figure 3.1C). After 72 hours in the light leaves were as turgid as untreated leaves, but had lost most of their chlorophyll. After 72 hours in the dark, leaves remain turgid but had lost far less chlorophyll than light-treated leaves.

**Effect of Humidity on Symptom Development**

The effect of humidity on symptom development was measured by incubating victorin treated leaves in a water-saturated atmosphere. Leaves were incubated in 100 ng/ml of victorin for 48 hr in either the light or dark. Leaves in the light are shriveled and completely desiccated, while leaves in the dark are yellowing but remain turgid (Figure 3.2A). By contrast, leaves incubated with 100 ng/ml toxin in the dark for 48 hr in a regular atmosphere are completely desiccated and shriveled (data not shown). Leaves incubated in normal or high humidity with 10 ng/ml of toxin for 48 hr showed a marked difference in symptom development (Figure 3.2B). Leaves in high humidity remained green and turgid after 48 hr, while leaves incubated under normal conditions are turgid but extensively yellowed.
**Effect of CO₂ on Symptom Development**

Leaves were incubated in the light with 100 ng/ml of victorin, for 24 hours in atmospheric conditions, or in air supplemented with 1% CO₂. 1% CO₂ lessens the effect of victorin (Figure 3.3A). Leaves remain turgid and show some chlorophyll loss. Protection against victorin by CO₂ is not complete however, and by 48 hrs, leaves treated with 100 ng/ml victorin in 1% CO₂ in the light are shriveled and desiccated (data not shown). The effect of 1% CO₂ on leaves treated with 10 ng/ml for 24 hr in the light is shown in Figure 3.3B. Leaves supplemented with 1% CO₂ are not showing any symptoms in response to 10 ng/ml of victorin. Leaves treated 100 ng/ml and 1% CO₂ in the dark appear more protected than leaves treated with 1% CO₂ in the light (Figure 3.3D). Five percent CO₂ appeared to give more protection to victorin-treated leaves than 1% CO₂ and protection seemed more durable than that given with 1% CO₂, although protection was not absolute at 5% CO₂ either. Leaves treated with 1 ng/ml victorin for 72 hr in the light with 5% CO₂, remain turgid and green and are only slightly shriveled at the edges (Figure 3.3 C), whereas leaves without CO₂ supplementation are slightly desiccated and shriveled, and are almost completely yellowed.

**CONCLUSION**

Recent evidence suggested that the site of action of victorin could be the glycine decarboxylase complex (Navarre and Wolpert, 1995). If GDC is the site of action, then one would anticipate that conditions which reduce photorespiration, might ameliorate the effect of victorin on sensitive plants. A simple bioassay for victorin is to detach leaves and incubate them in a victorin solution. The major victorin symptom using the leaf
bioassay and victorin concentrations equal or higher than 100 ng/ml is wilting, and within 24 hours leaf tissue is dead and virtually desiccated. Light has a major influence on symptom development, as shown in Figure 3.1. Leaves incubated with victorin in the dark are less affected than those incubated in the light. By 24 hours, light-incubated blades are desiccated and shriveled, whereas the dark-incubated leaves remain turgid and the major symptom is loss of chlorophyll (Figure 3.1A). Leaves incubated in increased CO₂ are protected against toxin. Five percent CO₂ gives near-complete protection against victorin, up to 24 hours after victorin treatment. By 48 hours, this protection has broken down and leaves die. Protection at 1% CO₂ also occurs, but is slightly less efficient than at 5%.

Several observations indicate that the protective effects of dark, humidity and CO₂ are not due to reduced rates of transpiration, which in turn might reduce toxin uptake: First, victorin is known to cause stomatal closure (Turner, 1972) which in turn, would minimize transpiration under any conditions. Furthermore, our own results show that stomates close within 30 minutes of treatment with 500 ng/ml of toxin (data not shown). Secondly, similar results are obtained when leaves are directly infiltrated with toxin which should circumvent any effects of altered respiration on toxin uptake. Finally, when leaves are incubated in glass vials, in which half of the leaf is within the vial containing the aqueous victorin solution (and thus, creating a small zone of higher humidity), the lower portion of the leaf displays a differential response to victorin. The portion of the leaf within the vial typically shows more yellowing and slower desiccation whereas the part of the blade protruding into the air rapidly shrivels. Thus, toxin is clearly being taken up by the upper portion of the leaf which is showing more severe symptoms. The
lessening of victorin effect in high humidity may have parallels to the decreased defense response in high humidity that has been reported in the tomato response to race-specific elicitors from Cladosporium (May et al., 1996)

The results presented in this paper support a role for the inhibition of glycine decarboxylase by victorin in disease development because leaves exposed to victorin under increased CO₂ or decreased light, conditions which reduce or eliminate photorespiration, protect against the effects of toxin. However, the phenotype of toxin-treated sensitive oat leaves under conditions of reduced photorespiration is not complete protection but rather a delayed response. Ultimately, sensitive oat leaves succumb to toxin treatment. Victorin is also apparently toxic to roots, a non-photorespiratory tissue, although root cells are less sensitive to victorin then leaf cells (Hawes, 1982). It has been demonstrated that GDC photorespiratory mutants are lethal when grown in normal atmosphere, but plants appear normal when grown under conditions of high CO₂ (Artus et al., 1994; Blackwell et al., 1990; Murray et al., 1989; McHale et al., 1988; Summerville and Ogren, 1982). These observations strongly suggest that GDC activity in plants is only required for photorespiratory activity. However, this response may reflect the growth condition under which all these mutants have been selected. For example, all of these mutants are somewhat "leaky" that is, they display a minimal amount of activity. It is possible that a non-leaky mutant would be lethal. Also, the conclusion that GDC is not required for functions other then photorespiration in plants seems inconsistent with the apparent role of GDC in non-plants. An essential role for the GDC apart from photorespiration is supported by the fact that the GDC is apparently found in all organisms for which it has been evaluated ranging from bacteria to humans and, thus, clearly has functions unrelated to
photorespiration. Defective GDC is the cause of hyperglycinemia, a lethal, incurable
disease in humans (Kume et al., 1988). The observation that glycine accumulates to toxic
levels suggests a substantial amount of carbon flow through the glycine-serine pathway in
humans. In *Escherichia coli* 15% of all carbon atoms assimilated from glucose are thought
to enter the glycine-serine pathway, and this pathway is also the major source of one-carbon
units in the cell (Wilson et al., 1993). The glycine-serine pathway is described as a major
metabolic pathway in non-plants (Stauffer, 1987) and thus, the same would seem likely in
the non-green tissues of plants. Also, the GDC is found in roots and etiolated tissues of oat
(Navarre and Wolpert, 1995) and other plants (cf. Walker and Oliver, 1986) and in C4
plants (Morgan et al., 1993), which suggests non-photorespiratory roles in plants. Thus,
given the possibility that GDC activity has an essential role in plants apart from
photorespiration, it may be expected that incubation of plants under non-photorespiratory
conditions would only provide partial protection against the effects of victorin. It is
possible however, that victorin might have additional sites of action other than GDC. We
cannot discount this possibility because of the lethality of victorin under non-
photorespiratory conditions.
Figure 3.1 Effect of Light and Dark on Symptom Development.

Leaves were detached and in either the light or dark with the treatment described. (A) Leaves incubated for 24 hr with 100 ng/ml of toxin, except for the leaves marked 0, which are the no toxin control. (B) Leaves incubated with 10 ng/ml for 24 hr. (C) Leaves incubated with 1 ng/ml toxin for 72 hr. (D) Leaves incubated with 10 ng/ml toxin for 48 hr.
Figure 3.2 Effect of Humidity on Symptom Development.

(A) Leaves were incubated in a water-saturated atmosphere, in either the light or dark, for 48 hr with 100 ng/ml victorin. (B) Leaves in light or dark were incubated in a water-saturated atmosphere for 48 hr with 10 ng/ml victorin.
Figure 3.3  Effect of CO₂ on Symptom Development.

(A) Leaves were treated with 100 ng/ml victorin for 24 hr with or without 1% CO₂.  (B) Leaves incubated with 10 ng/ml victorin for 24 hr with or without 1% CO₂.  (C) Leaves treated with 1 ng/ml victorin for 72 hr with or without 5% CO₂.  (D) Leaves incubated for 24 hr with 100 ng/ml victorin with 1% CO₂ in either the light or dark.
REFERENCES


INTRODUCTION

Victorin is a toxin produced by *Cochliobolus victoriae*, the fungus which causes victoria blight of oats (Meehan and Murphy, 1946). The discovery of victorin in the mid-1940s generated excitement because fungal cell-free culture filtrate diluted one million-fold was found to be not only toxic, but possessive of the same selective host specificity as the fungus (Meehan and Murphy, 1947). Thus, the toxin affects only oat cultivars susceptible to the fungus and no other known organism. The discovery of victorin led to the concept of 'host-selective' plant toxins and their subsequent study.

Victorin is required for *C. victoria* to successfully infect its host. Fungal strains which do not produce victorin are not pathogenic. Victorin also reproduces all the symptoms of the disease in the absence of the fungus. Sensitivity to victorin is dominant and is determined by a single plant gene, designated *Vb*. Therefore, the study of susceptibility and also host range is simplified to the study of the interaction of a single fungal metabolite, victorin, with the product of a single dominant plant gene, *Vb*.

The structure of victorin was not determined until 1985 when it was identified as a cyclized pentapeptide of 814 daltons (Wolpert et al., 1985). Subsequently, a biologically active I\(^{125}\)-victorin derivative was used to search for victorin-binding proteins in oat. Several binding proteins were found, one of which bound victorin in leaf slices from
susceptible but not resistant cultivars (Wolpert and Macko, 1989). The gene encoding this protein was cloned and identified as the P-protein component of the GDC complex, an important enzyme complex in photorespiration (Wolpert et al., 1994). Picomolar victorin concentrations inhibit GDC in leaf slices, while micromolar concentrations inhibit GDC activity 50% in vitro (Navarre and Wolpert, 1995).

Victorin research has focused on characterizing its role as a toxin. However, it may be simplistic to perceive victorin as strictly a classical toxin which simply kills cells, because victorin also induces plant responses classically caused by elicitors. Victorin effects such as callose production (Walton and Earle, 1985), respiratory burst (Romanko, 1959), ethylene evolution (Shain and Wheeler, 1975), extracellular alkalization (Ullrich and Novacky, 1991), phytoalexin synthesis (Mayama et al., 1986), and K⁺ efflux (Wheeler and Black, 1962) suggest victorin is a potent elicitor. Yet, despite its advantages, victorin has seldom been used to study such plant responses or the signal transduction chain involved, perhaps because victorin is typically conceptualized as a toxin. However, as host-selective toxins and race-specific elicitors become increasingly characterized, some of the distinctions between them become nebulous.

That victorin induces elicitor type plant responses is supported by the fact that victoria blight was first observed in the early 1940s when oat lines containing the \( P_{c-2} \) gene for superior rust disease resistance were released. These lines were devastated by victoria blight and it was determined that a single gene \( (V_b) \) was responsible for susceptibility. Extensive unsuccessful attempts were made to separate rust resistance from \( C. \ victoria \) susceptibility and these experiments strongly suggest that \( P_{c-2} \) and \( V_b \) are the same gene. Therefore, the same gene \( (V_b) \) may be responsible for resistance to
one disease (victoria blight) and susceptibility to another (crown rust). In this paper we present evidence that victorin induces a complex cascade which culminates in cell death and involves apoptosis. We characterize physiological effects of victorin, including a specific proteolytic cleavage of the Rubisco large subunit and describe parallels between victorin treated tissue and senescence.

METHODS

Plants and Materials.

Oat seedlings were grown in a growth chamber for 5-7 days under a 16 hr photoperiod at 24 °C. The resistant oat line x424, and susceptible line x469 were used. Chemicals used for signal transduction studies were purchased from Sigma or Calbiochem.

Characterization of Cleaved Rubisco Large Subunit

For a typical assay of Rubisco cleavage, 10 leaf slices of ca. 2 mm were incubated in a final volume of 100 ul at 25 °C in either the dark or light for the time indicated. The solution in which the leaf slices were incubated was removed and 200 ul of water-saturated phenol and a small amount of sand added. Leaf slices were then homogenized in microcentrifuge tubes, after which 200 ul of homogenization buffer (50 mM Tris, 0.7 M sucrose, 5 mM DTT, 100 mM KCl, 5 mM EDTA, pH 7.0) was added and the two phases mixed. Samples were centrifuged in a microcentrifuge for 2 minutes at 12,000 rpm and the phenol phase collected. One ml of 0.1M ammonium acetate in methanol was added to the phenol phase and proteins precipitated for at least 2 hours at -20 °C. Samples were centrifuged for 5 minutes at 12,000 rpm in a microcentrifuge and the
resulting pellet rinsed once with methanol. The pellet was resuspended in 100 ul of sample buffer (6 M urea, 2% w/v SDS, 60 mM Tris, 20 mM DTT, pH 6.8) and typically 10-20 ul of this sample was loaded onto a 14% SDS-PAGE gel and analyzed by electrophoresis. To check whether a cleaved LSU product was associated with thylakoid membranes, leaf slices were treated for 4 hours in the dark with 100 ng/ml victorin. Leaf slices were then homogenized in 50 mM Tris, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 0.1 mM E-64. The homogenate was centrifuged for 15 minutes at 12000 rpm in a microcentrifuge. The resulting pellet was considered the thylakoid fraction and the supernatant the stromal fraction.

Detection of Lipid Peroxidation

Malondialdehyde was assayed essentially as described by Dhindsa et al., 1981. Twenty leaf slices were incubated in either the dark or light for the time indicated.

Purification of Rubisco

Thin (ca. 1 mM) leaf slices from 20 blades of 7-day-old plants were incubated in the dark at 25 °C for 4 hours with 100 ng/ml victorin. The leaf slices were rinsed with water and homogenized in 20 ml of 20mM Tris-HCl, 5 mM EDTA, 2 mM DTT, 1 mM PMSF, 0.1 mM E-64, 0.1 mM leupeptin, pH 8.0 with a chilled mortar and pestle. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 5,000 rpm for 5 min. The supernatant was collected and centrifuged at 60,000 x g for 60 min at 4 °C. The resulting pellet was discarded and the supernatant loaded directly onto a C4 HPLC column (vyadec) equilibrated in Buffer A (2% MeOH, 0.2% TFA). Protein was then eluted with a linear gradient of 0-70% ACN in Buffer A. Consecutive fractions were
analyzed by SDS-PAGE. The purified Rubisco fraction was then electrophoresed on a 14% SDS gel and the gel electroblotted onto Immobilon as previously described (Navarre and Wolpert, 1995). The N-terminal amino acid sequence was determined on an Applied Biosystems, Model 475A gas-phase protein sequencer operated by the Central Service Lab of the Center for Gene Research and Biotechnology (Oregon State University).

**DNA Extraction**

The epidermal layer was peeled from leaves, which were then floated on a 100 ng/ml victorin solution for 3-6 hours. Approximately ten 10 cm leaf segments were used per treatment and incubated in the dark at room temperature with slow shaking. Treated leaves were frozen in liquid nitrogen and a CTAB procedure used to extract DNA. DNA laddering was observed using DNA separated on a 2% agarose gel, stained with ethidium bromide and photographed with a BioPhotronics Gelprint 2000i imaging system (BioPhotronics, Ann Arbor, MI).

**RESULTS**

**Victorin Generates a Protein Doublet**

We were interested in further characterizing the physiological effects of victorin on sensitive plants, and examining whether we could detect evidence that the diverse effects of victorin are mediated by a signal transduction system. We noticed the appearance of a ca. 55 kDa protein doublet when leaf slices were treated with victorin.
Figure 4.1 Effect of victorin on a 55 kDa protein.

(A) Coomassie-blue stained SDS-polyacrylamide gel. Leaf slices were incubated in the dark for 4 hr with the indicated victorin concentration. Protein was then extracted and separated on a 14% gel. (B) A stained SDS-polyacrylamide gel containing total protein from leaf slices treated for 4 hr with or without 100 ng/ml victorin. lane 1, no toxin; lane 2, 100 ng/ml; lane 3, no toxin, 2.5 times as much protein as lane 1; lane 4, 100 ng/ml, 2.5 times as much toxin as lane 2.
Total leaf slice protein was extracted directly into phenol and the proteins separated via SDS-PAGE. Figure 4.1A shows the appearance of this doublet in leaf slices incubated in the dark for 4 hours with different victorin concentrations. The protein doublet is first detectable in leaf slices treated with 10 ng/ml of victorin (lane 4). Tissue treated with 100 ng/ml has an increased amount of the lower protein band and a decreased amount of the upper band. The combined intensity of the Coomassie blue-stained doublet matched the intensity of the single 55 kDa protein prior to appearance of the doublet. The upper protein band almost completely disappeared in tissue treated with 1000 ng/ml of victorin and the smaller 53 kDa protein increased (lane 6). This suggested that the lower protein band was not due to the synthesis of a new protein, but rather to either cleavage of the 55 kDa protein or a modification which altered its mobility. This protein appeared to be the Rubisco large subunit (LSU) because of its 55 kDa size, its localization to chloroplasts (data not shown) and because it is the most abundant protein present in total protein extracts. This identification was later confirmed by protein sequencing (described later in this paper).

This effect on LSU seemed very specific and not due to general cellular degeneration because other proteins appeared unaltered as judged by the protein profile on stained gels (Figure 4.1B). Total leaf proteins from untreated and treated leaf slices were extracted after 3 hours and deliberately overloaded on SDS-PAGE gels so that less abundant proteins could be better visualized. The protein profile appears identical between victorin-treated and untreated samples in the overloaded lanes (3 and 4) except for the effect on the Rubisco large subunit. A slight distortion of 3-4 bands clustered
immediately underneath the Rubisco band is evident in treated tissue, but this could be
due to the compression caused by the appearance of large amounts of the cleaved LSU.

**Characterization of the Rubisco Modification**

The alteration in LSU mobility could be due to a non-proteolytic modification of
the protein that alters its mobility or it could be due to proteolytic cleavage. Proteinases
localized within chloroplasts have been described (Musgrove et al., 1989; Bushnell et all.,
1993). To distinguish between these possibilities, leaf slices treated with victorin were
co-incubated with the protease inhibitors E-64 or leupeptin. Figure 4.2A shows that both
protease inhibitors prevented cleavage of LSU in vivo. E-64 concentrations as low as 10
uM completely prevented cleavage and the first trace of cleavage was not detectable until
E-64 concentrations of 1 uM (lane 5). Leupeptin inhibits both serine and cysteine
proteases, whereas E-64 is thought to be specific for cysteine proteases. This suggested a
cysteine protease is responsible for cleavage of the Rubisco large subunit.

We compared the effect of light versus dark on victorin-induced LSU cleavage.
Leaf slices were treated with various victorin concentrations for 6 hrs to generate a dose
response curve and one set of samples was incubated in the light and another set
concurrently in the dark. Figure 4.2B shows that the effect of victorin on LSU is
markedly different in the light. Light seems to accelerate the loss of LSU. However, no
LSU doublet was ever observed at any tested victorin concentration when samples were
incubated in the light. Possibly, the cleaved product is degraded too fast to accumulate
detectable amounts in the light, or perhaps a different mechanism of LSU breakdown is
operational in the light.
Other investigators have observed rapid Rubisco degradation in the light (Casano and Trippi, 1992; Mehta et al., 1992; Mitsuhashi et al., 1992). Casano et al., (1994) found proteolysis in isolated oat chloroplasts incubated in photo-oxidative conditions, but negligible proteolysis in the dark unless the chloroplasts were oxidatively stress.

Leaf slices were treated with 100 ng/ml of victorin in light or dark for different time intervals to generate a time course. In the dark, cleavage first becomes noticeable between 2 and 4 hours as shown in Figure 4.2C. Cleavage continues in a time-dependent manner until LSU is completely cleaved. The cleaved product appeared surprisingly stable in the dark. Even 18 hours after treatment with victorin (lane 6) the cleaved product was still present in high amounts. In contrast, victorin treated leaf slices incubated in the light showed a rapid disappearance of LSU without accumulating visible amounts of the cleaved LSU at any time point. LSU degradation in dark incubated tissue appeared to proceed to the formation of a stable intermediate, whereas the LSU in light incubated tissue appears to undergo complete proteolytic degradation. After 6 hours, treated tissue in the dark still has roughly as much total LSU present (cleaved and uncleaved) as untreated tissue (lane 4), whereas LSU levels are greatly decreased in victorin treated tissue after 6 hours in the light (lane 4). After 18 hours of incubation in the light discrete protein bands are still detectable, but these bands contain only a small amount of the total protein initially present in each band, whereas after 18 hours in the dark, leaf tissue protein was far less degraded. Thus, these data also show that victorin is more lethal in the light than dark as might be expected given victorin's inhibition of the glycine decarboxylase complex.
Figure 4.2 Effect of protease inhibitors or light on Rubisco effect.

(A) Protein gel showing the effect of protease inhibitors on victorin-treated leaf slices. E-64 was used at the indicated concentrations in lanes 3, 4 and 5, and leupeptin in lane 6. (B) Comparison of the effect of light vs dark on Rubisco cleavage. Leaf slices were treated with the indicated victorin concentrations for 6 hr and total leaf protein extracted and separated on protein gels. (C) Time course of the effect of victorin on Rubisco cleavage in leaf slices treated with 100 ng/ml victorin for the time indicated in the light or dark.
Involvement of Calcium in the Victorin Response

Given the numerous effects of victorin on plants, it seemed possible that victorin initiated a signal transduction cascade in susceptible plants, leading to the diverse effects observed. To begin to delineate this chain of events we used various signal transduction antagonists in an attempt to block or alter the effect of victorin. LaCl₃, a calcium channel blocker not thought to enter cells, appeared to give complete protection against victorin as shown in Figure 4.3A. Leaves treated with 10 mM LaCl₃ remained symptom free for several days. Leaf slices treated with victorin and LaCl₃ did not undergo Rubisco cleavage (Figure 4.3B, lane 3). EGTA also was partially effective at preventing LSU cleavage (lane 4), as cleavage occurred but was less than the control. Other calcium channel blockers such as verapamil and nifidipine (lane 5 and 6) also gave a degree of protection, but were considerably less effective than LaCl₃. These data suggest that extracellular calcium is at least partially involved in the transduction of the victorin initiated signal, though this does not preclude a role for organellar calcium. Treatment with ruthenium red, a putative blocker of organellar calcium channels supported this interpretation, because it was only partially effective in preventing LSU cleavage. Limited protection with ruthenium red was variable, as some experiments showed no visible protection. Calpeptin is an inhibitor of calpain, a calcium activated cysteine protease, and it prevented LSU cleavage when present in nanomolar concentrations. A Ca++ or Mg++ dependent chloroplast stromal protease has been described which degrades LSU in vitro, but not other tested chloroplastic proteins (Bushnell et al., 1993).
Figure 4.3. Effect of calcium on symptom development.

(A) Panel A,B,C: Leaves treated for 24 hr with 100 ng/ml victorin and the treatment indicated. (B) Protein gel showing total leaf protein extracted from leaf slices that were incubated for 4 hours with victorin and the indicated treatment.
Ethylene and Symptom Development

Victorin treated leaf slices are known to evolve ethylene (Shaine and Wheeler, 1975). Two different ethylene inhibitors, AOA and STS were used to examine the possible role of ethylene in the development of symptoms in victorin treated plants. Leaves were pretreated with AOA or STS for 1 hour, and then 100 ng/ml of victorin was added and plants incubated in the light for 24 hours. Both AOA and STS delay the development of symptoms as seen in Figure 4.3A, panel B. AOA appears to give slightly better protection than STS. Neither compound gives total protection against victorin, unlike LaCl₃, but they do markedly slow the development of victorin symptoms. Both an inhibitor of ethylene action (STS) and ethylene synthesis (AOA) were effective in preventing Rubisco cleavage in leaf slices (Figure 4.6A, lane 4 and 5). Others have shown that blocking ethylene synthesis or action in leaves prevents chlorophyll loss and protein degradation associated with senescence (Aharoni et al., 1979; Gepstein and Thimann, 1981). Thus, ethylene, in addition to calcium, appears needed to transduce the signal which leads to LSU cleavage.

Identification of the LSU Cleavage Site

Rubisco was purified from victorin-treated leaf slices incubated for 4 hours in the dark. Very thin leaf slices (ca 1mm) were used because minimal uncleaved LSU remains in the tissue after victorin treatment. This facilitated separation of the cleaved product from uncut LSU. Cleaved LSU was purified by a combination of SDS-PAGE and HPLC. The N-terminal amino acid sequence of the cleaved protein was determined and the sequence revealed that the cleavage is N-terminal and occurs at lysine 14. Figure 4.4
shows the amino acid sequence obtained compared to the protein sequence from spinach, tobacco and rice. LSU is apparently vulnerable to cleavage at lysine 14 because other investigators have found that in vitro treatment of Rubisco with trypsin or endopeptidase Lys C cleaved at lysine 14 causing complete loss of CO₂ fixing ability without destroying the quaternary structure (Gutteridge et al., 1986; Mulligan et al., 1987). As a control to identify any artifactual proteolytic problems during the purification process, the Rubisco large subunit was also purified by the same purification procedure from untreated leaf slices incubated 4 hours in the dark. In this case LSU was not sequenceable, presumably because the N-terminus is blocked, as is reported in the literature. Thus, artifactual proteolysis during purification was not problematic.

**Victorin Causes DNA Cleavage**

Because victorin treated leaf tissue has parallels with senescing tissue, such as chlorophyll loss and Rubisco degradation, and data suggested the possibility that victorin triggers a cascade that culminates in cell death, we examined the effect of victorin treatment on plant DNA. Leaves, with their epidermal layers peeled away to facilitate victorin uptake, were treated with 100 ng/ml of victorin for 3 or 6 hours and DNA was then extracted and separated on a 2% agarose gel. As shown in Figure 4.5, the DNA has been cleaved into a ladder. This type of DNA cleavage pattern is the hallmark of apoptotic cell death. As discussed earlier, calcium seems to be important in the plant
Cleaved Rubisco was purified from victorin treated leaf slices and the amino acid sequence of the first 31 N-terminal amino acids determined. The amino acid residues 14 through 45 from 3 other plant species are presented for comparison with the amino acid sequences of 3 other plant species.
response to victorin, and calcium is also involved in apoptosis in mammalian systems and also in DNA laddering in response to AAL toxin in tomato (Wang et al., 1996). Furthermore, a calcium-dependent nuclease has been identified in rice chloroplasts during senescence (Sodmergen et al., 1991).

**Effect of Various Treatments on LSU Cleavage**

Cleavage of the Rubisco large subunit was additionally characterized by incubating leaf slices with various compounds before or during victorin treatment. A few studies have found that Rubisco becomes tightly associated with the thylakoids during oxidative stress (Mehta et al., 1992) or after fruit removal in soybean (Crafts-Brandner et al., 1991), but this does not appear to be the case in victorin treated leaf slices. Tissue was homogenized and separated into a pellet and supernatant fraction and analyzed by SDS-PAGE. LSU was found in the supernatant, not the thylakoid fraction (Figure 4.6A, lanes 7 and 8). DTT was also found to prevent LSU cleavage (Figure 4.6A, lane 9). Another treatment which prevented LSU cleavage was CO₂ (Figure 4.6B). We had previously shown that whole plants incubated in increased CO₂ had partial protection against victorin (Chapter 3). Figure 4.6B shows that leaf slices incubated with bicarbonate concentrations as low as 10 mM did not undergo Rubisco cleavage. Bicarbonate protection deteriorates at 1 mM, where a small amount of cleaved LSU is seen. Spermine was effective at blocking LSU cleavage (Figure 4.6A, lane 6) and although its mode of action is unknown, polyamines block senescence, inhibiting proteolysis, chlorophyll degradation and ethylene evolution (Kaur-Sawhney and Galston, 1988). Interestingly,
Figure 4.5 Victorin induced DNA cleavage.

DNA was extracted from peeled leaf slices incubated with or without 100 ng/ml victorin for the times shown. DNA was separated on a 2% agarose gel and stained for visualization. Progressively smaller concentrations of DNA are loaded from left to right across the gel.
ZnCl\(_2\) prevented Rubisco cleavage (lane 3). Zinc is known to prevent apoptosis in mammals and it also prevented AAL-toxin induced apoptosis in tomato (Want et al., 1996). ZnCl\(_2\) also delayed the effect of victorin at the whole plant level. Leaves treated with 10 mM ZnCl\(_2\) remained healthy 24 hrs after treatment with 100 ng/ml victorin (Figure 4.3A). No effect on Rubisco was observed when a resistant genotype of oat (424) was incubated with victorin concentrations as high as 100 \(\mu\)g/ml (data not shown).

NAP, a phosphatase inhibitor, seemed to worsen the effect of victorin on LSU. 5mM NAP causes LSU cleavage in the dark, in the absence of toxin (Figure 4.6C). This is the only molecule we tested that mimicked the effect of victorin on LSU. LSU cleavage does not occur when leaf slices are treated with 1 ng/ml of victorin for 4 hours. Furthermore, at 1mM NAP only a trace amount of cleaved LSU is visible. However, when leaf slices are simultaneously treated with 1 mM NAP and 1 ng/ml of victorin, considerable LSU cleavage resulted (Figure 4.6C), suggesting the two compounds may have synergistic effects.

A 30 min exposure of leaf slices to 100 ng/ml of victorin is enough to induce LSU cleavage. LSU cleavage occurred in victorin-treated leaf slices that were rinsed after 30 min and then incubated without victorin in the dark for 3.5 hrs (Figure 4.6D). Lanthanum is effective in blocking LSU cleavage when added 1 hr after victorin treatment in such treated and rinsed leaf slices. LaCl\(_3\) prevents LSU cleavage when added as late as 2 hours after toxin (data not shown). This suggests that either a sustained calcium flux may be needed, or that a calcium signal occurs upstream of LSU cleavage and is not induced until several hours after victorin treatment. KCN also prevents LSU cleavage when added 1 hour after victorin (Figure 4.6D) as does ZnCl\(_2\).
Figure 4.6 Effect of various treatments on Rubisco cleavage by victorin.

(A) Protein gel containing total protein from leaf slices incubated for 4 hours in the dark with the treatments indicated. Protein was extracted and separated using SDS-PAGE. (B) Protein gel containing total protein from leaf slices treated with victorin and the indicated concentrations of bicarbonate for 4 hours in the dark. (C) Protein gel containing total protein from leaf slices incubated 4 hours in the dark with the indicated treatment. (D) Protein gel containing total protein from leaf slices pretreated for 30 minutes with 100 ng/ml of victorin and then rinsed and after another 30 minutes the indicated treatment was added. Leaf slices were then incubated in the dark for 3 additional hours.
Table 4.1 lists treatments that were ineffective in preventing toxin-induced LSU cleavage in leaf slices or causing LSU cleavage (Bombesin, Glycine Hydroxymate, CuNO₃). Many of these compounds have been shown effective in blocking various plant responses to elicitors in cell culture suspensions, but had no effect here. All compounds listed were tested at a minimum of 3 different concentrations, varying at least a thousand-fold. The efficacy of their uptake by leaf slices, as opposed to cell cultures is unknown. Leaf slice pretreatments of up to 5 hours with cycloheximide or kanamycin did not prevent Rubisco cleavage (data not shown). This suggests that the protease involved is probably activated, as opposed to translationally regulated.

Detection of Lipid Peroxidation

Victorin inhibits the photorespiratory cycle and therefore should increase the oxidative load on plants. Victorin effects include chlorophyll loss, photobleaching and Rubisco degradation and such effects in other plants are often associated with lipid peroxidation and generation of active oxygen species. Figure 4.7A shows that leaf slices treated with various victorin concentrations in the light, accumulated malondialdehyde (MDA), a by-product of lipid peroxidation. MDA levels increased over 300% in leaf slices treated with 100 ng/ml victorin. MDA was not detected in victorin treated leaf

Several compounds which prevent LSU cleavage by victorin were tested to see whether they also prevented lipid peroxidation. LaCl₃ prevented lipid peroxidation (Figure 4.7B), but the protease inhibitor E-64, which prevents LSU cleavage did not. Indeed, E-64-treated tissue, consistently produced more MDA than tissue treated only
Table 4.1 Compounds tested for an effect on Rubisco cleavage

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mode of Action</th>
<th>Compound</th>
<th>Mode of Action</th>
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<tbody>
<tr>
<td>Staurosporine</td>
<td>Broad spectrum protein kinase inhibitor</td>
<td>Genestein</td>
<td>Protein Kinase inhibitor</td>
</tr>
<tr>
<td>K-252A</td>
<td>General protein kinase inhibitor</td>
<td>R24571</td>
<td>Calmodulin antagonist</td>
</tr>
<tr>
<td>Catalase</td>
<td>$\text{H}_2\text{O}_2$ metabolism</td>
<td>SQ22536</td>
<td>Adenylate cyclase inhibitor</td>
</tr>
<tr>
<td>2-Ethyl-2-thiopseudourea</td>
<td>Inhibits nitric oxide synthase</td>
<td>CuNO$_3$</td>
<td>Causes oxidative stress</td>
</tr>
<tr>
<td>Bisindolylmaleimide I</td>
<td>Protein Kinase C inhibitor</td>
<td>Kinetin/zeatin</td>
<td>Cytokinins</td>
</tr>
<tr>
<td>Bombesin</td>
<td>Ins(1,4,5)P3-induced Ca$^2+$ and K$^+$ fluxes</td>
<td>Glycine Hydroxymate</td>
<td>Inhibits Glycine Decarboxylation</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Dismutates superoxide</td>
<td>Tetraethylammonium chloride</td>
<td>$K^+$ channel blocker</td>
</tr>
</tbody>
</table>
Figure 4.7 Malondialdehyde is Generated by Victorin Treatment.

Leaf slices were incubated with the indicated victorin concentrations for 4 hr in the light. MDA was then extracted, quantified and expressed as the percent of MDA accumulation in untreated leaf slices. (B) Leaf slices were incubated with the treatment indicated and with 100 ng/ml victorin for 4 hours in the light, except for the single dark treatment. MDA was then extracted and quantified and expressed as percent of MDA accumulation in untreated leaf slices.
with victorin. E-64 also failed to provide any visible protection to detached leaves incubated with victorin. Thus, lipid peroxidation does not appear to be a consequence of Rubisco cleavage. Spermine, AOA and STS all reduced the amount of MDA detected, but did not provide complete protection. Calpeptin was more effective than E-64 at reducing the amount of MDA generated (Figure 4.7B).

**Effect of Respiratory Inhibitors on LSU Cleavage**

Because victorin inhibits glycine decarboxylase, a mitochondrial enzyme complex, we were interested in any link between mitochondria and Rubisco cleavage, because presumably the initial effect of victorin is to inhibit glycine decarboxylation. KCN concentrations greater than 1 mM prevent LSU cleavage by victorin. Leaf slices treated with 1mM KCN and victorin, show a minimal amount of LSU cleavage (Figure 4.8, lane 3). Sodium Azide was also effective in preventing LSU cleavage, as was DNP, an uncoupler of phosphorylative oxidation. Oligomycin at 5 µg/ml gave partial protection against LSU cleavage (lane 5). Rotenone was ineffective, but could only be tested at low concentrations because of solubility problems. Antimycin concentrations at 50 µg/ml failed to prevent LSU cleavage by victorin.

**DISCUSSION**

**Victorin Induces a Signal Cascade**

As more is learned about race-specific elicitors and host-selective toxins, the distinctions between them become nebulous at times. For example, a particularly potent elicitor would have the selectivity and lethality of host-selective toxins. Though usually
**Figure 4.8** Effect of Respiratory Inhibitors on Rubisco Cleavage by Victorin.

Protein gel containing total protein from leaf slices incubated for 4 hours in the dark with the treatment indicated.
conceptualized as a toxin, victorin clearly induces many plant responses classically associated with elicitors. The data presented in this paper show that victorin causes diverse effects in sensitive plants. The near-complete protection against victorin by LaCl3 (Figure 4.3A) was particularly interesting for several reasons. No compound had previously been found which protected against victorin for several days, or when administered to the plant at the same time as victorin. Furthermore, the LaCl3 protection against victorin indicates that victorin does not directly kill sensitive plants as would be expected for a classic toxin, but rather the plant itself apparently mounts a response to victorin that results in self-destruction. This is consistent with other data in this paper that suggest victorin induces a signal transduction cascade in the plant. Finally, the LaCl3 data implicates calcium as a second messenger in the plant response to victorin. One possibility we will examine in future research is that victorin induces a calcium flux. Lending support to a possible calcium flux is that fact that most, if not all, of the effects caused by victorin have been linked to calcium in other systems. Stomatal closure, KCl efflux, lipoxygenase activity, respiratory burst, callose synthesis, DNA degradation, ethylene evolution, senescence (Poovaiah, 1987) and phytoalexin synthesis all may involve calcium in some instances. Ethylene also appears involved in transducing the signal which leads to LSU cleavage, because ethylene inhibitors prevent LSU cleavage and provide partial protection against victorin at the whole leaf level (Figure 4.3A and 4.6A). Phosphatases are also implicated in the victorin induced cascade because NAP, a phosphatase inhibitor, worsens the effect of victorin. NAP is also the only compound we found that could cause LSU cleavage in leaf slices in the absence of victorin (Figure 4.6C). This suggests phosphorylation and dephosphorylation events may mediate the
victorin response, however none of the protein kinase inhibitors we tested (Table 1) prevented victorin induced LSU cleavage.

Rubisco Cleavage

One interesting victorin effect is a specific cleavage of the Rubisco large subunit at lysine-14, 14 amino acids from the N-terminus (Figure 4.4). Considerable literature exists on Rubisco degradation, particularly in terms of plant senescence. Plants have exquisite control over the degradative process of Rubisco, initiating these processes in response to various environmental signals such as cold stress, ozone (Landry and Pell, 1993) jasmonate (Weidhase et al, 1987) osmotic stress (Ferreira and Davies, 1989) low CO₂ (Ferreira and Davies, 1989), and oxidative stress (Casano et al., 1990; Mehta et al., 1992).

Rubisco cleavage products have been reported many times in the literature, primarily from in vitro studies. For example, cleavage of the Rubisco LSU can occur during purification (Paech and Dybing, 1986). Also, during treatment with exogenous proteases, the purified Rubisco large subunit is shortened by 1-2 kDa without destroying the L₈S₈ quaternary structure (Gutteridge et al. 1986). Most reports of cleaved LSU products have not come from studies of intact tissue, but rather from experiments using chloroplasts or purified enzyme. A 37 kDa LSU fragment has been detected in isolated chloroplasts incubated in the dark, and degradation was accelerated in the light (Mitsuhashi et al., 1992). Mehta et al. (1992) detected LSU fragments in oxygen-stressed, isolated chloroplasts, but not in intact plants in which no intermediates were detected during LSU degradation, possibly due to faster rates of degradation. The single
example we are aware of detection of a Rubisco fragment in whole tissue was reported for bean leaf disks aged in the dark, in which a 45 kDa breakdown product was detected (Hildbrand, et al., 1994).

Little is known about the transduction chain leading to Rubisco degradation in any plant, nor the mechanism by which cleavage occurs. Victorin apparently activates this unknown mechanism, thereby causing LSU cleavage. Victorin induced LSU cleavage at lysine 14 suggests this mechanism of Rubisco regulation may be significant in planta and is not merely an artifact of in vitro systems where cleavage at lysine-14 has been repeatedly observed. Rubisco is inactivated by cleavage at lysine-14 (Gutteridge et al., 1986). Curiously, lysine-14 often has a post-translational trimethylation modification as shown by Houtz et al., (1992) who found lysine-14 contained the modification in 8 of 10 species examined. The role of the lysine trimethylation is unknown, though it has been speculated to protect against proteolytic degradation in the case of a lys-115 methylation of calmodulin (Gregori et al., 1987).

**Parallels Between Victorin Effects and Senescence**

The symptoms caused by victorin in some ways resemble an accelerated senescence. Rubisco degradation is one of the hallmarks of senescence, as is chlorophyll loss. In certain conditions, such as high humidity, the most noticeable victorin symptom is a near-total loss of chlorophyll and the plant remains turgid (Chapter 3). Lipid peroxidation also occurs in victorin-treated tissue (Figure 4.7), and is also prominent in senescence. Leaf senescence is the classic example of programmed cell death, and victorin treated leaves have the pronounced DNA ladder characteristic of
apoptosis—a word which means leaf senescence (Figure 4.5). Ethylene is involved in senescence and ethylene is synthesized in response to victorin. Furthermore, ethylene inhibitors prevent Rubisco cleavage by victorin. Polyamines are also known to prevent or delay senescence, and spermine was effective at preventing LSU cleavage (Figure 4.6A). CO₂ is known to delay senescence and elevated CO₂ concentrations also delay the effect of victorin (Chapter 3) on whole plants and detached leaves, and bicarbonate prevents LSU cleavage (Figure 4.6B). However, interpretation of the protective effect of CO₂ is complicated, because elevated CO₂ delays senescence, but also prevents photorespiration. Thus, by decreasing photorespiration CO₂ could minimize the consequences of inhibition of glycine decarboxylation by victorin. Whether victorin is initiating a true senescence is impossible to say at this point, because the biochemical and molecular basis of plant senescence is poorly characterized, nor are universal markers unique to senescence known.

There are some interesting linkages between senescence and photorespiration in the literature. Significantly, barley and arabidopsis glycine decarboxylase mutants undergo premature senescence. Widholm and Ogren (1969) showed that C3 plants placed under photorespiratory conditions underwent premature senescence, whereas C4 plants did not. Mondal and Choudhuri (1982) showed that leaf slices from C3 species senesced faster than C4 plants. Satler and Thimann (1983) showed either high O₂ or CO₂-free air accelerated senescence in oat, whereas 2% O₂ or 1% CO₂, delayed senescence. Martin and Thimann (1972) examined the effect of various amino acids on senescence in oats and found serine and cysteine promoted senescence. Both serine and cysteine are known to inhibit GDC. Thus, the literature suggests in some instances there
may be a linkage between photorespiration and senescence. The plant response to
victorin is consistent with a linkage between photorespiration and senescence, particularly
when using victorin concentrations which are not instantly lethal, thus giving symptoms
time to develop.

That photorespiratory stress may be involved in some instances of senescence is
not surprising since oxidative stress in general is associated with the induction of
senescence. Mehta et al., (1992) suggested that the cellular and stromal oxidative state
may initiate senescence. Thimann and Satler (1979) found that treatments which caused
oat stomates to close, accelerated the loss of chlorophyll and proteolysis (senescence).
Stomatal closure in sunlight can lead to superoxide production and photoinhibition and
photooxidative damage (Scandalios, 1993). Victorin causes stomates to close and this
should place the plant under even greater oxidative stress in the light, because stomatal
closure will decrease the intracellular CO$_2$ concentration and cause increased
photorespiration. Thus, victorin not only causes sensitive plants to be in a state which
favors O$_2$ fixation (by closing stomates), victorin then prevents the photorespiratory
pathway from coping with the increased photorespiration by inhibiting glycine
decarboxylation. Consequently, victorin should cause leaves in the light to be under
increased oxidative stress. Victorin causes lipid peroxidation (Figure 4.7) which is often
associated with the production of free radicals and victorin also causes photobleaching.
Oxidative stress is likely important in the victorin-induced Rubisco cleavage. H$_2$O$_2$ and
oxygen radicals increase proteolytic activity in isolated oat chloroplasts (Casano et al.,
1990; Casano and Trippi, 1992). Penarrubia and Moreno (1990) showed that oxidative
treatments of purified Rubisco increase its susceptibility to trypsin and chymotrypsin
generating a doublet similar to that seen in victorin treated tissue. Mehta et al., (1992) showed a highly reduced environment must be maintained in the chloroplast to prevent S-S cross-linking within Rubisco and they speculated that stresses which increase the stroma’s oxidative state may trigger Rubisco degradation and senescence. Furthermore, H₂O₂ concentrations as low as 25 uM have been shown to inhibit enzymes of the Calvin cycle and can cause peroxidation of vital thiol groups (Robinson, 1988). Many Calvin cycle enzymes are regulated by the redox state of their thiol’s which are reductively activated by thioredoxin. A role for thiols in the victorin response is suggested by the prevention of Rubisco cleavage in treated leaf slices by DTT (Figure 4.7A), which may protect by keeping stromal thiols in a reduced state. An electron microscopy study of leaf tissue treated with paraquat, a generator of oxygen free radicals, showed extensive alterations in the Rubisco holozyme prior to alterations in the chloroplast ultrastructure (Fraser et al., 1994).

**Victorin and Chloroplast Function**

The effect of victorin on chloroplastic function occurs downstream of its initial effect on the cell. Victorin does inhibit light-dependent CO₂ fixation in leaf slices, but this inhibition occurs later than the inhibition of glycine decarboxylation and is less sensitive to victorin than is glycine decarboxylation (Navarre and Wolpert, 1995). If Rubisco is no longer able to utilize the NADPH produced by photosynthesis, then that NADPH may be available for use elsewhere in the cell, including by NADPH oxidases. The role of chloroplasts in the development of victorin symptoms is potentially interesting. For example, what is the fate of electrons generated by the photosynthetic
apparatus if the Calvin cycle is non-operative or chloroplasts are losing integrity? Given the large number of chloroplasts present in leaves, a disruption in chloroplast electron transport would likely generate large amounts of free radicals in sunlight. Such a mechanism could initiate lipid peroxidation, and is consistent with lipid peroxidation being detected in victorin treated leaf slices incubated in the light, but not the dark. Furthermore, O₂ concentration amongst all organisms is the highest in plants, and is found in leaf cells at over 250 uM, as compared to 0.1 uM near mammalian mitochondria (Scandalios, 1993). Moreover O₂ concentrations of up to 300 uM may occur in the chloroplast (Robinson, 1988). Thus, any perturbation by victorin of chloroplast function, has the potential to produce significant amounts of active oxygen species, which would be highly detrimental to the leaf.

As mentioned, the protective effect of LaCl₃ (Figure 4.3A) suggests cell death occurs as a result of actions initiated by the plant in response to victorin and that whatever the initial effect of victorin is, this effect must be transduced before cell death results. The apoptotic DNA cleavage observed also suggests that programmed cell death may be occurring. Victorin may force the plant into a premature senescence, or at least victorin mimics senescence. Theoretically, plants have a very effective, rapid and tightly regulated method of triggering cell death if the plant initiates chloroplast degradation. The role of chloroplasts in hypersensitive cell death observed in response to pathogens is also interesting, although not much studied despite some intriguing observations. Purohit and Tregunna (1975) found that 1% CO₂ inhibited lesion production in tobacco plants inoculated with two viral pathogens and they speculated about a photorespiratory link. Peever and Higgins (1989) found that tomato plants treated with a specific elicitor
preparation from *Cladosporium* underwent necrosis in the light, but not in the dark. Light is also required for generation of a hypersensitive response by rice to *Xanthomonas* (Guo, et al., 1993). Light is also required for toxin induced necrosis in tomato leaflets treated with AAL toxin which, like victorin, is a host-selective toxin (Moussatos, et. al., 1993). T-toxin, also a HST, has a mitochondrial site of action yet causes rapid chlorophyll loss in the light, but not the dark (Bhullar, et al., 1975). Like victorin, T-toxin inhibits photosynthesis and causes stomatal closure (Arntzen, et al., 1973).

It is difficult not to speculate on the possible role of an accelerated senescence in a host-pathogen interaction. From the plant’s perspective one can visualize a situation where it is in the plant’s interest to prematurely enter senescence by sacrificing a part of its biomass, recycling the nutrients from this senescing tissue and at the same time depriving the pathogen of living cells and sustenance. On the other hand, the pathogen could benefit by causing the host tissue to self-destruct, thereby negating any host defenses in those cells and at the same time freeing up a rich pool of nutrients. Such a strategy might be to the plants advantage with biotrophic pathogens, and to the pathogen’s advantage with necrotrophic pathogens.

**Victorin and Glycine Decarboxylation**

Is the binding of victorin by GDC the initial step in the cascade described in this paper? If the binding of victorin by GDC is the crucial event, how does inhibiting GDC lead to all of the secondary effects described? We do know that victorin is a potent
inhibitor of GDC and it is known that GDC mutants undergo early senescence. The
precise details of why the barley and arabidopsis GDC mutants senesce are not known, but presumably this senescence is a result of oxidative stress resulting from the plant’s inability to cope with photorespiration. In this instance, the mitochondrial GDC inactivity leads to chloroplastic, and presumably, cell-wide events. Thus, there is a precedence for some of the effects seen with victorin. Furthermore, CO₂ lessens the toxicity of victorin, which would seem to implicate the GDC. Finally, pyridoxal and pyridoxal phosphate prevent cleavage of LSU by victorin. This is interesting because pyridoxal phosphate competes with binding of victorin by the P-protein of victorin (Wolpert, unpublished data). However, it is possible that victorin directly interacts with some other cellular component in addition to GDC, and that it is this interaction responsible for some of victorin’s effects. Whatever the initial event, it seems likely that both oxidative stress and a calcium flux play a central role in the plant response to victorin. Future research that would help delineate the victorin-induced signal transduction cascade is the use of confocal microscopy to search for the occurrence of a calcium flux in victorin treated tissue. Another interesting research area is the examination of other plant leaf tissue undergoing a hypersensitive response, for similar chloroplastic effects as described in this paper. Better characterization of the signal cascade leading to cell death in victorin treated tissue will further clarify how victorin induces the numerous diverse plant responses observed, and might also allow one to work backwards towards the initial event triggering the cascade, thus, complementing ongoing studies of the direct effect of victorin on GDC.
REFERENCES


Chapter 5

CONCLUSION

Victoria blight of oats is caused by the fungus *Cochliobolus victoriae*. This fungus is pathogenic due to its ability to produce the host-selective toxin victorin. Previously, a 100-kD protein that binds victorin in vivo only in susceptible genotypes was identified as the P protein of the glycine decarboxylase complex (GDC). The work described in this dissertation suggests that the interaction of victorin and GDC may be central to the toxin’s mode of action. Evidence summarized below suggests the interaction of victorin and GDC is important:

1. Victorin binds the P-protein in vivo, only in susceptible genotypes.
2. Victorin is a potent in vivo inhibitor of GDC, displaying an effective concentration for 50% inhibition (EC$_{50}$) of 81 pM for GDA. Concentrations of victorin as low as 5 pM inhibit GDC.
3. Victorin inhibits the glycine-bicarbonate exchange reaction in vitro with an EC$_{50}$ of 23 µM. Thus, victorin inhibits the purified enzyme also. This strongly argues against the effect of victorin on GDA being non-specific, or the indirect result of general cellular decay due to toxin treatment.
4. Victorin also binds the H protein component of the GDC. Thus, victorin specifically binds to two components of the GDC.
5. Very high doses of victorin result in 20-30% inhibition of GDA in resistant genotypes. This is significant because highly pure victorin is not known to affect resistant genotypes.
6. All known forms of victorin have either a glycine or glyoxylate residue, both of which are intermediates of the photorespiratory cycle.
6. All known forms of victorin have either a glycine or glyoxylate residue, both of which are intermediates of the photorespiratory cycle.

7. Green tissue is more sensitive to victorin than roots or epidermal tissue. This is significant because green tissue has higher amounts of GDC than non-green tissue. Green tissue is also the only tissue which photorespires.

8. Elevated CO₂ gives partial protection against victorin. Oats grown in elevated CO₂ will have less photorespiration and thus be less affected by inhibition of GDC.

9. Victorin treated plants incubated in the light develop more severe symptoms than dark incubated plants.

10. Victorin appears to accumulate in mitochondria from susceptible, but not resistant plants. GDC is a mitochondrial enzyme complex.

11. Pyridoxal gives partial protection against many of the effects of victorin. Pyridoxal is a cofactor of the P-protein and was found to compete with victorin for binding by the P-protein.

Victorin appears to induce a plant wide signal transduction cascade, resulting in diverse effects. Many of the effects caused by victorin are also seen in other plant species that have been treated with race-specific elicitors. For example, victorin treated tissue has a K⁺ efflux, callose synthesis, ethylene evolution, phytoalexin synthesis, respiratory burst, extracellular alkalization and lipid peroxidation. As more is learned about host-specific elicitors, parallels are becoming increasingly obvious with some host-selective toxins and the distinction between host-selective toxins and race-specific elicitors becomes less distinct. One novel effect of victorin is a specific proteolytic cleavage of the Rubisco large subunit (LSU), 14 amino acids from the N-terminus. Cleavage of the Rubisco LSU has been observed many times in vitro by various investigators. However, the cleavage of LSU by victorin occurs in vivo and suggests that this type of Rubisco cleavage is not just an artifact of in vitro systems and may have biological significance.
The LSU cleavage observed after victorin treatment does not appear just to be an artifact of victorin treatment, or of Rubisco purification for the following reasons:

1. The cleavage is very specific and all other proteins observed in stained SDS-PAGE gels seem unaltered.

2. LSU purified by the identical procedure from untreated plants did not have the cleaved form of LSU.

3. LaCl3 and KCN prevented cleavage of LSU in victorin treated tissue, even when added 1-2 hours after the toxin. This suggests an active process is involved, and also implies that upstream events must occur in the victorin induced signal transduction cascade prior to LSU cleavage.

Depending on the victorin concentration used and environmental conditions, the most visible symptom of victorin treatment is chlorophyll loss. Thus, two of the primary symptoms of victorin treated plants are chlorophyll loss and Rubisco LSU cleavage. These two symptoms (chlorophyll loss and protein degradation) are also hallmarks of senescence. As discussed in Chapter 4, other data, such as DNA laddering and ethylene synthesis also suggest victorin is inducing a premature senescence in sensitive oats. Most, if not all, of the effects of victorin may possibly be mediated by an alteration in the plant’s calcium homeostasis. Almost all of the effects of victorin discussed above, particularly those shared with race-specific elicitors, have been linked to calcium fluxes in other systems. Consistent with calcium being important in regulating the plants response to victorin is the complete or near-complete protection against victorin conferred by LaCl3, a calcium channel blocker.

In addition to calcium, oxidative stress is likely very important in the toxicity of victorin: Victorin causes stomates to close, which will results in increased photorespiration and increased oxidative stress on plants in the light. Furthermore, at the same time victorin inhibits the plants ability to cope with photorespiration, because victorin inhibits GDC. Thus, victorin not only puts the plant in a state which favors
photorespiration, victorin also inhibits the plants ability to survive photorespiratory stress. Thus, in the light considerable oxidative stress is likely to be occurring in victorin treated plants, from the consequences of stomatal closure in sunlight, inhibition of GDC and LSU cleavage. The Rubisco LSU cleavage is likely to result in inhibition of the Calvin cycle and disruption of chloroplast electron flow. Thus it is possible that large amounts of free radicals may be produced in the chloroplasts of victorin treated plants in the light. Oxygen radicals play a pivotal role in mammalian disease interactions, and increasing evidence implicates oxygen radicals as key mediators of plant host-pathogen interactions. The results presented in this thesis suggest that a particularly interesting avenue of future research in plant biology may be analysis of the role of chloroplasts in host-pathogen interactions. What are the consequences to both plant and pathogen as chloroplast function is impaired? Each cell has 100s of chloroplasts which are capable of producing free radicles from light, particularly as normal chloroplast electron flow is disrupted. Thus, plants potentially have a unique mechanism for generating large quantities of free radicals in the light, a mechanism not shared by mammals. Furthermore, chloroplasts have oxygen concentrations of up to 300 uM as opposed to mammalian mitochondria which have oxygen concentrations of 0.1 uM. Thus, chloroplasts have an electron and oxygen pool in close proximity that is not matched in mammalian systems.

One speculative interpretation of the data presented in this thesis is that when invaded by a pathogen, plants may ‘choose’ to prematurely trigger localized senescence in the infected region. By sacrificing an infected leaf by initiating senescence in that leaf, the plant may deprive the pathogen of needed living cells or nutrients, while at the same time recycling part of its own biomass, thereby managing to reclaim some of its resources. From the pathogen’s perspective, it may be to the pathogen’s advantage to force the plant into senescence, thereby creating a pool of nutrients and also circumventing plant defense mechanisms that require living plant cells.
In conclusion, the interaction of victorin and GDC appears to be important, quite possibly pivotal, to the mode of action of victorin. A component of the GDC could be the product of the Vb gene. Because victorin may interact with each of the 4 proteins which comprise the GDC complex, any one of the proteins could be the Vb gene product. However, a GDC protein could be the site of action of victorin, without being the product of the Vb gene. An example of such a scenario would be if another protein regulates victorin’s access to the GDC complex. Alternatively, a GDC protein could be the product of the Vb gene, but not victorin’s site of action. In this case, victorin would be metabolized by the GDC complex and the resulting metabolite of victorin could be toxic elsewhere in the cell.

Finally, despite substantial evidence pointing to the importance of victorin’s inhibition of the GDC, we cannot discount the possibility that victorin may have another site of action in the cell which has no relation to the GDC. The primary observation which suggests this is a possibility is the fact that victorin is toxic in the dark—although less so than in the light. Photorespiration does not occur in the dark, and the requirement for GDC by the plant is thought to be essential only when photorespiration is occurring. Delineating this point is the fact that barley and arabidopsis GDC mutants grow normally when grown in an atmosphere supplemented with CO₂, but die when transferred to normal atmospheres which allows photorespiration to occur. However, these mutants may not have been null mutants. If GDC is the sole target of victorin, then GDC must have an essential role in the plant in addition to its role in photorespiration. The alternative explanation of course, is that victorin has an additional site of action in the cell. Even if this is the case, the inhibition of GDC by victorin is still likely important in disease development and symptomology and may account for the greater lethality of victorin in the light as compared to the dark.


