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Victorin Induction of an Apoptotic/Senescence–like Response in Oats

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Victorin is a host-selective toxin produced by *Cochliobolus victoriae*, the causal agent of victoria blight of oats. Previously, victorin was shown to be bound specifically by two proteins of the mitochondrial glycine decarboxylase complex, at least one of which binds victorin only in toxin-sensitive genotypes in vivo. This enzyme complex is involved in the photorespiratory cycle and is inhibited by victorin, with an effective concentration for 50% inhibition of 81 pM. The photorespiratory cycle begins with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and victorin was found to induce a specific proteolytic cleavage of the Rubisco large subunit (LSU). Leaf slices incubated with victorin for 4 hr in the dark accumulated a form of the LSU that is cleaved after the 14th amino acid. This proteolytic cleavage was prevented by the protease inhibitors E-64 and calpeptin. Another primary symptom of victorin treatment is chlorophyll loss, which along with the specific LSU cleavage is suggestive of a victorin-induced, senescence-like response. DNA from victorin-treated leaf slices showed a pronounced laddering effect, which is typical of apoptosis. Calcium appeared to play a role in mediating the plant response to victorin because $LaCl_3$ gave near-complete protection against victorin, preventing both leaf symptoms and LSU cleavage. The ethylene inhibitors aminooxyacetic acid and silver thiosulfate also gave significant protection against victorin-induced leaf symptoms and prevented LSU cleavage. The symptoms resulting from victorin treatment suggest that victorin causes premature senescence of leaves.

INTRODUCTION

Victorin is a toxin produced by *Cochliobolus victoriae*, which is the fungus that causes victoria blight of oats (Meehan and Murphy, 1946). Victoria blight was first described in the mid-1940s. Considerable interest in the disease was generated by the novel observations that cell-free, fungal culture filtrate diluted one millionfold was not only toxic but exhibited the same specificity toward oat genotypes as the fungus (Meehan and Murphy, 1947). The discovery of victorin led to the concept of "host-selective" plant toxins and their subsequent study.

Victorin is required for *C. victoriae* to infect its host successfully. Fungal strains that do not produce victorin are not pathogenic. Victorin also reproduces the disease symptoms in the absence of the fungus. Oat 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 oat gene, *Vb.*

Victorin research through the decades has focused almost exclusively on characterizing its role as a toxin. However, it may be simplistic to perceive victorin as strictly a toxin that kills cells, because victorin also induces plant responses classically associated with elicitors, such as callose production (Walton and Earle, 1985), a 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). Victorin seldom has been used to study such plant responses or the signal transduction chain involved, perhaps because victorin typically is conceptualized as a toxin.

The dual elicitor/toxin nature of victorin is illustrated by the fact that victoria blight never had been observed before the release in the 1940s of oat lines containing the *Pc-2* gene for rust disease resistance. These lines were devastated by victoria blight. Extensive unsuccessful attempts have been made to separate rust resistance from *C. victoriae* susceptibility, and these experiments suggest that *Pc-2* and *Vb* are the same gene or are closely linked (Rines and Luke, 1985; Mayama et al., 1995). Therefore, the same gene (*Vb*) may be responsible for susceptibility to one disease (victoria blight) but resistance to another (crown rust).

The structure of the most prevalent form of victorin has been identified as a cyclized pentapeptide of 814 D (Wolpert et al., 1985). A biologically active, I^{125}-victorin derivative was produced and used to search for oat victorin binding proteins. Several binding proteins were found, one of which

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binds 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 glycine decarboxylase complex (GDC), an important enzyme complex in the photorespiratory cycle (Wolpert et al., 1994). Picomolar victorin concentrations inhibit GDC in leaf slices, and micromolar concentrations inhibit GDC activity in vitro (Navarre and Wolpert, 1995).

We do not understand how or whether the inhibition of GDC by victorin leads to all of the various known effects of victorin. One possibility is that the GDC has other functions in addition to its involvement in the photorespiratory cycle (discussed in Navarre and Wolpert, 1995). A distinctly different possibility is that victorin has additional targets besides the GDC. With the hope of gaining insight into the mode of action of victorin, we attempted to characterize further the physiological and biochemical effects of victorin on susceptible plant tissue. Such analysis might clarify the significance of the inhibition of the GDC by victorin. Because victorin inhibits the GDC, a photorespiratory cycle enzyme, and because ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) initiates the photorespiratory process, we were intrigued particularly by the occurrence in victorin-treated tissue of an alteration of the Rubisco large subunit (LSU) (this study). We were interested in whether alteration of the Rubisco LSU was a metabolic feedback effect resulting from GDC inhibition. We characterize a specific proteolytic cleavage of the Rubisco LSU and describe parallels between victorin-treated tissue and senescence. We describe an elicitor-like, victorin-induced response that culminates in cell death and involves apoptosis.

RESULTS

Victorin Generates a Protein Doublet

When leaf slices were treated with victorin, an ~55-kD protein doublet appeared in protein gel analyses. Leaf slices were directly homogenized in phenol, a denaturant, to minimize proteolysis during extraction. The SDS–polyacrylamide gel in Figure 1A shows the appearance of this doublet in leaf slices incubated in the dark for 4 hr with various victorin concentrations. The protein doublet was detectable in leaf slices treated with as little as 10 ng/mL victorin (Figure 1A, fourth lane). Tissue treated with 100 ng/mL had an increased amount of the lower protein and a decreased amount of the upper protein. The upper protein almost completely disappeared in tissue treated with 1000 ng/mL of victorin, and the smaller 53-kD protein increased (Figure 1A, sixth lane). The combined Coomassie Brilliant Blue R 250–stained intensity of each band in samples showing a doublet matched the intensity of the single 55-kD protein in controls. This suggests that the lower protein was not due to the synthesis of a new protein but rather to either a proteolytic cleavage of the 55-kD protein or some other dose-dependent, victorin-induced modification that altered its mobility. This protein appeared to be the Rubisco LSU because of its size of 55 kD and its localization to chloroplasts (data not shown), and because it was the most abundant protein

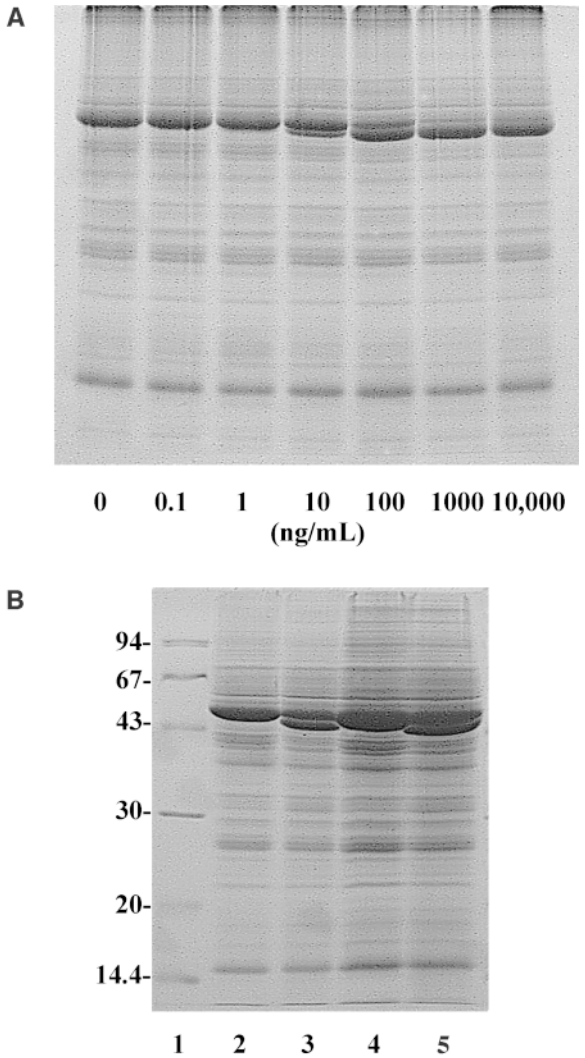

Figure 1. Effect of Victorin on a 55-kD Protein.

(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 contains molecular mass markers given in kilodaltons; lane 2, protein from leaf slices treated with no toxin; lane 3, protein from leaf slices treated with 100 ng/mL victorin; lane 4, protein from leaf slices treated with no toxin, with 2.5 times as much protein as lane 2; and lane 5, protein from leaf slices treated with 100 ng/mL victorin, with 2.5 times as much protein as lane 3.

present in total protein extracts. This identification was confirmed by protein sequencing as described later in this study.

This effect on the LSU seemed very specific and not due to general cellular degeneration because other proteins appeared unaltered, as determined by the protein profile of stained gels (Figure 1B). Total leaf protein from untreated and treated leaf slices was extracted after 4 hr and deliberately overloaded on SDS–polyacrylamide gels so that less abundant proteins could be visualized better. The protein profile between victorin-treated and untreated samples in the overloaded lanes (Figure 1B, lanes 4 and 5) appeared identical, except for the effect on the Rubisco LSU. A slight distortion of three or four bands clustered immediately underneath the Rubisco band is evident in treated tissue, but this is likely due to the compression caused by the appearance of large amounts of the altered LSU. No effect on the protein profile of victorin-treated resistant oat tissue was detected.

Characterization of the Rubisco Modification

The alteration in LSU mobility could be due to a nonproteolytic modification of the protein that alters its mobility or it could be due to proteolytic cleavage. Proteinases localized within chloroplasts have been described previously (Musgrove et al., 1989; Bushnell et al., 1993). To distinguish between these possibilities, leaf slices treated with victorin were coincubated with the protease inhibitors E-64 or leupeptin. Figure 2A shows that both protease inhibitors prevented cleavage of the LSU in vivo. E-64 concentrations as low as 10 μM completely prevented cleavage, and the first trace of cleavage was not detectable until the E-64 concentration was lowered to 1 μM (Figure 2A, lane 5). Leupeptin inhibits both serine and cysteine proteases, whereas E-64 is thought to be specific for cysteine proteases. This suggests that a cysteine protease is responsible for cleavage of the Rubisco LSU.

We compared the effect of light versus dark on victorin-induced LSU cleavage. Leaf slices were treated with various victorin concentrations for 6 hr to generate a dose–response profile, and one set of samples was incubated in the light and another set concurrently in the dark. Figure 2B shows that the effect of victorin on the LSU is markedly different in the light. No LSU doublet was ever observed at any tested victorin concentration when samples were incubated in the light. However, there was a dose-dependent decrease in the amount of LSU protein detectable. Possibly, the cleaved product was degraded too quickly to accumulate to detectable amounts in the light, or perhaps a different mechanism of LSU breakdown was 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 photooxidative conditions but

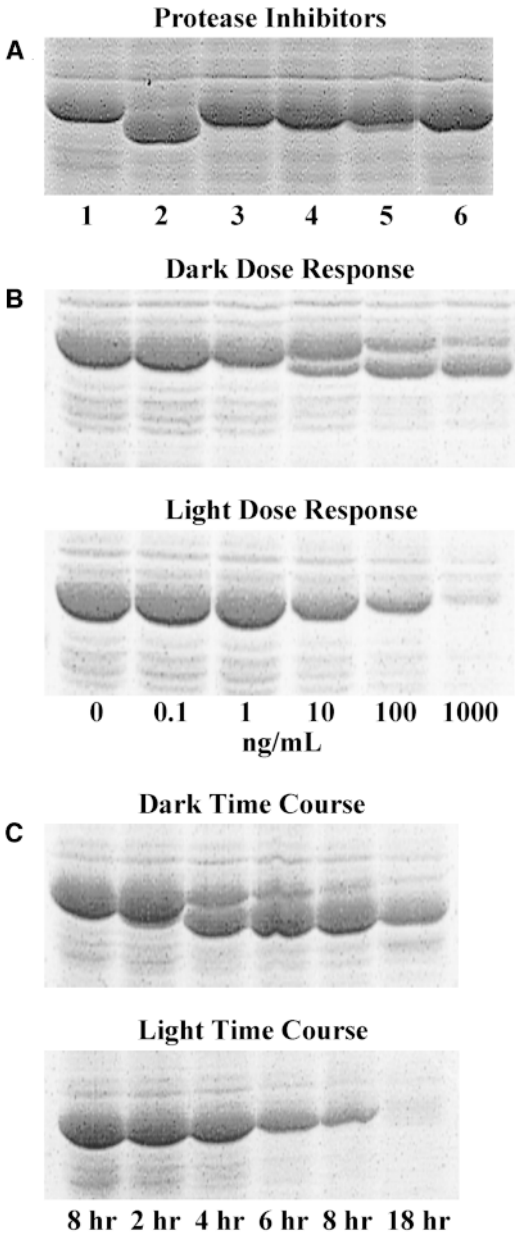

Figure 2. Effect of Protease Inhibitors or Light on Rubisco Mobility.

(A) Protein gel showing the effect of protease inhibitors on leaf slices treated with victorin. Lane 1, no toxin treatment; lane 2, treatment with 100 ng/mL victorin; lanes 3 to 5, treatment with 100 ng/mL victorin plus 100, 10, and 1 μM, respectively, E-64; and lane 6, 100 ng/mL victorin and 100 μM leupeptin.

(B) The dose–response effect of victorin treatment on Rubisco cleavage in dark versus light. Leaf slices were treated with the indicated victorin concentrations for 6 hr, and total leaf protein was extracted and separated on 14% protein gels.

(C) Time course of the effect of victorin on Rubisco cleavage. The first lane contains protein from leaf slices incubated for 8 hr without victorin. The remaining lanes contain protein from leaf slices incubated with 100 ng/mL victorin in the light or dark for the time indicated.

negligible proteolysis in the dark unless the chloroplasts were oxidatively stressed.

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 became noticeable between 2 and 4 hr, as shown in Figure 2C. Cleavage continued in a time-dependent manner until the LSU was completely cleaved. The cleaved product appeared surprisingly stable in the dark. Even 18 hr after treatment with victorin (Figure 2C, sixth lane), the cleaved product was still present in high amounts. In contrast, victorin-treated leaf slices incubated in the light showed a rapid disappearance of the 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 appeared to undergo complete proteolytic degradation. After 8 hr, treated tissue in the dark (Figure 2C, fifth lane) still has approximately as much total LSU present (cleaved and uncleaved) as untreated tissue (Figure 2C, first lane), whereas LSU levels are greatly decreased in victorin-treated tissue after 8 hr in the light (fifth lane). After 18 hr 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 hr in the dark, leaf tissue protein was far less degraded. Thus, these data show that victorin's effects are more severe in the light than in the dark, as might be expected given victorin's inhibition of the GDC.

Identification of the LSU Cleavage Site

The altered, putative Rubisco LSU was purified from leaf slices incubated with victorin for 4 hr in the dark. Thin leaf slices ($\sim$1 mm) were used to maximize the cleaved LSU in the tissue after victorin treatment, which facilitated separation of the cleaved product from the uncleaved LSU. Cleaved Rubisco LSU was purified to homogeneity by a combination of reversed-phase HPLC and SDS-PAGE. The N-terminal amino acid sequence of the cleaved protein confirmed that the protein was the Rubisco LSU and that cleavage was at the N terminus and occurred after the 14th amino acid. Figure 3 shows the amino acid sequence obtained from oat compared with the Rubisco LSU amino acid sequence starting at lysine-14 from spinach, tobacco, and rice (Hudson et al., 1990). The 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 CO_2 fixing ability without destroying the quaternary structure (Gutteridge et al., 1986; Mulligan et al., 1988).

As a control, to identify any potential, artifactual proteolytic cleavage during the purification process, the Rubisco LSU also was purified by the same purification procedure from untreated leaf slices incubated 4 hr in the dark. In this case, the LSU could not be sequenced, presumably because the N terminus was blocked, as has been reported in the literature (Houtz et al., 1992). The cleaved Rubisco LSU purified by HPLC had mobility in SDS–polyacrylamide gels identical to the lower band of the protein doublet observed when leaf proteins were extracted directly into phenol (data not shown). In addition, E-64 prevented cleavage when added to leaf slices concurrently with victorin (Figure 2A) but not when added to leaf slices after victorin treatment but before extraction (data not shown). Thus, artifactual proteolysis during purification was not problematic.

Victorin Causes DNA Cleavage

Two hallmarks of senescence in plants are chlorophyll loss and Rubisco degradation. With high concentrations of victorin ($>$100 ng/mL), the major symptom of victorin-treated oat leaves is typically severe wilting, but at lower victorin concentrations, leaves remain turgid, and the major symptom is a total loss of chlorophyll (D.A. Navarre and T.J. Wolpert, unpublished data). The loss of chlorophyll along with the specific Rubisco degradation suggests the possibility that victorin triggers a response similar to senescence, a form of programmed cell death (Greenberg, 1996). We examined the effect of victorin treatment on plant DNA to determine whether the DNA laddering characteristic of apoptosis was present. Leaves with epidermal layers peeled away to facilitate victorin uptake were treated for 3 or 6 hr with 100 ng/mL of victorin, and DNA was then extracted and separated on a 2% agarose gel. As shown in Figure 4, the DNA was cleaved into discrete sizes, typically described as laddering. Similar results were obtained when leaf slices were used, but the quality of high molecular weight DNA in controls was better with whole leaves.

Involvement of Calcium in the Victorin Response

This evidence for victorin-induced apoptosis suggests that victorin might be triggering a signal transduction cascade in

OAT:	AGVKDYKLTY YTPEYETKDT DILAAFRVTPQ
Spinach	KAGVKDYKLTY YTPEYET**L**DT DILAAFRV**S**PQ
Tobacco	KAGVK**E**YKLTY YTPEY**Q**TKDT DILAAFRVTPQ
Rice	KAGVKDYKLTY YTPEYETKDT DILAAFRV**S**PQ

Figure 3. Amino Acid Sequence of the N Terminus of the Cleaved Protein.

Cleaved Rubisco was purified from victorin-treated leaf slices, and the amino acid sequence of the first 31 N-terminal amino acids was determined. Amino acid residues 14 through 45 from three other plant species are presented for comparison with the amino acid sequence of the protein extracted from victorin-treated oats. Bold, small capital letters indicate residues that are divergent from the consensus sequence.

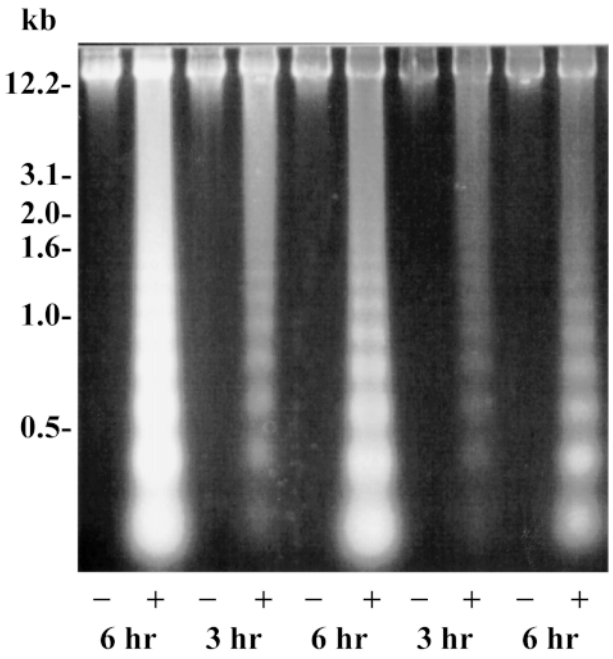

Figure 4. Victorin-Induced DNA Cleavage.

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 visualized by ethidium bromide staining. Progressively smaller concentrations of DNA from the same samples taken at 3 or 6 hr were loaded from left to right across the gel. The numbers at the left indicate the relative migration of DNA length standards.

susceptible plants. If such a cascade exists, it could explain the diverse effects that victorin has on plants, such as the senescence-like effects and the elicitor-like effects of victorin discussed earlier. 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. Because calcium has been implicated in some forms of mammalian apoptosis (Martin et al., 1994), we examined the possible role of calcium in victorin-treated tissue. $LaCl_3$, a calcium channel blocker not thought to enter cells (Hall, 1987), appeared to give near-complete protection against victorin, as shown in Figure 5. Leaves treated with 10 mM $LaCl_3$ remained symptom free for several days. Leaf slices treated with victorin and $LaCl_3$ did not undergo Rubisco cleavage (Figure 6, lane 3). EGTA also was partially effective in preventing LSU cleavage (Figure 6, lane 4). Cleavage occurred but was less extensive than in the toxin-treated control. Other calcium-channel blockers, such as verapamil and nifidipine (Figure 6, lanes 5 and 6), also gave a degree of protection but were considerably less effective than was $LaCl_3$.

These data suggest that extracellular calcium is at least partially involved in the transduction of the victorin-initiated signal, but they do 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, because in some experiments, no visible protection was observed. Calpeptin is an inhibitor of calpain, a calcium-activated cysteine protease, and it prevented LSU cleavage when present in nanomolar concentrations (Figure 6) and was a more effective inhibitor of LSU cleavage than was either E-64 or leupeptin (Figure 2A). Thus, victorin-mediated LSU cleavage may be due to a calcium-dependent activation of a cysteine protease. A Ca^{2+}- or Mg^{2+}-dependent chloroplast stromal protease has been described that degrades the LSU in vitro but not other tested chloroplastic proteins (Bushnell et al., 1993).

Ethylene and Symptom Development

Victorin-treated leaves are known to evolve ethylene (Shain and Wheeler, 1975). Silver thiosulfate (STS), an inhibitor of ethylene action, and aminooxyacetic acid (AOA), an ethylene synthesis inhibitor, were used to examine a possible role for ethylene in the development of symptoms in victorin-treated plants. Detached leaves were pretreated with AOA or STS for 1 hr, and then 100 ng/mL of victorin was added and plants were incubated in the light for 24 hr. Both AOA and STS delayed symptom development, as seen in Figure 5 (leaf sets 4 and 5). AOA appeared to give slightly better protection than did STS. Neither compound gave the level of protection against victorin that was provided by $LaCl_3$, but they did markedly slow the development of victorin symptoms. Both STS and AOA were effective in preventing Rubisco cleavage in leaf slices (Figure 7A, lanes 4 and 5). Others have shown that blocking ethylene synthesis or action in leaves prevents the chlorophyll loss and Rubisco degradation associated with senescence (Aharoni et al., 1979; Gepstein and Thimann, 1981). Thus, ethylene, in addition to calcium, appears to participate in the process that leads to LSU cleavage.

Effect of Various Treatments on LSU Cleavage

In a few studies, Rubisco was found to become 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. Victorin-treated tissue was homogenized in a non-osmotic buffer, separated into a pellet and a supernatant fraction, and analyzed by SDS-PAGE. The LSU was found in the supernatant (stroma) but not in the thylakoid fraction (Figure 7A, lanes 7 and 8).

Cleavage of the Rubisco LSU was characterized further by incubating leaf slices with various compounds before or

Figure 5. Protection against Victorin-Induced Symptom Development.

Leaves were treated for 1 hr with the agents indicated, followed by the addition of 100 ng/mL victorin (or water for the water control) for 24 hr.

during victorin treatment. DTT was found to prevent LSU cleavage (Figure 7A, lane 9). Another treatment that prevented LSU cleavage was CO_2 (Figure 7B). We previously found that whole plants incubated in increased CO_2 exhibited partial protection against victorin (D.A. Navarre and T.J. Wolpert, unpublished data). Figure 7B shows that leaf slices incubated with bicarbonate concentrations as low as 10 mM did not undergo Rubisco cleavage. Bicarbonate protection deteriorated at 1 mM; at this concentration, a small amount of the cleaved LSU was seen. Spermine was effective at blocking LSU cleavage (Figure 7A, lane 6). Spermine's mode of action is not known, but polyamines block senescence, inhibiting proteolysis, chlorophyll degradation, and ethylene evolution (Kaur-Sawhney and Galston, 1986). Interestingly, $ZnCl_2$ prevented Rubisco cleavage (Figure 7A, lane 3). Zinc is known to prevent apoptosis in mammals, and it also prevented AAL toxin–induced apoptosis in tomato (Wang 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 hr after treatment with 100 ng/mL victorin (Figure 5).

Naphthyl acid phosphate (NAP), a phosphatase inhibitor, seemed to enhance the effect of victorin on the LSU. At 5 mM, NAP caused LSU cleavage in the dark in the absence of toxin (Figure 7C, lane 4). NAP is also the only compound we found that could cause LSU cleavage in leaf slices in the absence of victorin. LSU cleavage does not occur when leaf slices are treated with 1 ng/mL victorin for 4 hr. Furthermore, at 1 mM NAP, only a trace amount of the cleaved LSU was visible. However, when leaf slices were treated simultaneously with 1 mM NAP and 1 ng/mL victorin, considerable LSU cleavage resulted (Figure 7C), suggesting that the two compounds may have synergistic effects. The NAP data suggested that phosphorylation and dephosphorylation events may mediate the victorin response. However, none of the protein kinase inhibitors that we tested, including staurosporine, genistein, and bisindolylmaleimide I (data not shown), prevented victorin-induced LSU cleavage.

A 30-min exposure of leaf slices to 100 ng/mL victorin is sufficient to induce LSU cleavage. LSU cleavage occurred in leaf slices that were treated with victorin for 30 min followed by removal of victorin by rinsing and dark incubation without victorin for 3.5 hr (Figure 7D, lane 3). Lanthanum was effec-

tive in blocking LSU cleavage when added 1 hr after victorin treatment in treated and rinsed leaf slices (Figure 7D, lane 4). Indeed, $LaCl_3$ prevented LSU cleavage when added as late as 2 hr after the toxin (data not shown). This suggests that either a sustained alteration in calcium homeostasis may have been induced or the calcium signal was not induced until several hours after victorin treatment. KCN also prevents LSU cleavage when added 1 hr after victorin (Figure 7D, lane 1), as does $ZnCl_2$ (Figure 7D, lane 2).

Leaf slice pretreatments of up to 5 hr with cycloheximide or kanamycin did not prevent Rubisco cleavage (data not shown). This suggests that the protease involved is likely activated, as opposed to translationally regulated.

Detection of Lipid Peroxidation

Victorin inhibits the photorespiratory cycle and therefore should increase the oxidative load on plants in the light. In other plants, effects such as chlorophyll loss, photobleaching, and Rubisco degradation are often associated with lipid peroxidation and generation of active oxygen species (Dhindsa et al., 1981). Figure 8 shows that leaf slices treated with various victorin concentrations in the light accumulated thiobarbituric acid–reactive species (TBARs), a marker of lipid peroxidation. TBAR levels increased >260% in leaf slices treated with 100 ng/mL victorin. TBARs were not detected in victorin-treated leaf slices incubated in the dark. This is additional evidence that victorin's effects are more severe in the light than in the dark. Furthermore, more TBARs were generated in response to victorin as the light intensity increased (data not shown).

Several compounds that prevent LSU cleavage by victorin were tested to determine whether they also prevented lipid peroxidation. $LaCl_3$ prevented lipid peroxidation (Figure 8). The protease inhibitor E-64 prevented LSU cleavage but was ineffective in preventing lipid peroxidation. E-64 also failed to provide any visible protection to detached leaves

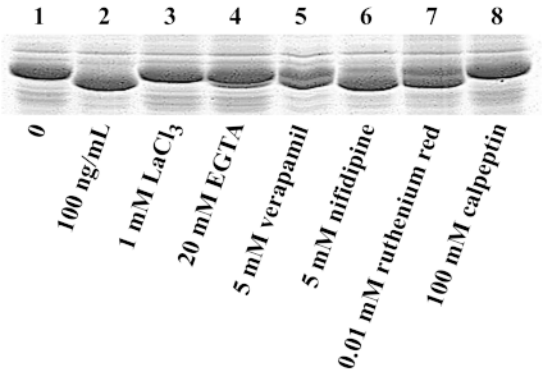

Figure 6. Effect of Calcium on Victorin-Induced Rubisco Cleavage.

Leaf slices were incubated with victorin and the indicated inhibitors for 4 hr. Total protein was then extracted and separated on a protein gel. Lane 1 contains protein from leaf slices treated with no victorin; lane 2, protein from leaf slices treated with 100 ng/mL victorin; the remaining lanes contain protein from leaf slices treated with 100 ng/mL victorin plus the indicated inhibitors.

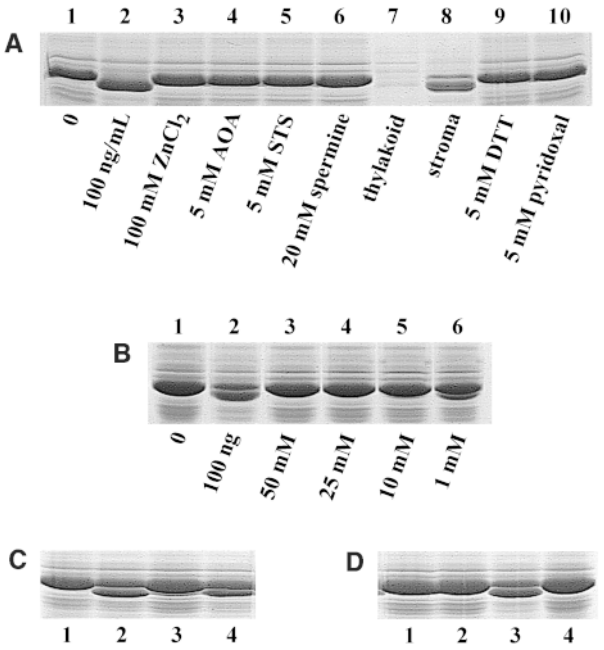

Figure 7. Effect of Various Agents on Victorin-Induced Rubisco Cleavage.

(A) Protein gel containing protein from leaf slices incubated for 4 hr in the dark with the agents indicated. Protein was extracted and separated on a 14% SDS–polyacrylamide gel. Lane 1 contains protein from leaf slices treated with no victorin; lane 2, protein from leaf slices treated with 100 ng/mL victorin; and lanes 3 to 10, protein from leaf slices treated with 100 ng/mL victorin plus the agent indicated or extracted from the fractions described.

(B) Protein gel containing total protein from leaf slices treated with victorin and the indicated concentrations of bicarbonate for 4 hr in the dark. Lane 1 contains protein from leaf slices treated with no toxin; lane 2, protein from leaf slices treated with 100 ng/mL victorin; and lanes 3 to 6, protein from leaf slices treated with 100 ng/mL victorin plus the indicated amount of bicarbonate.

(C) Protein gel containing total protein from leaf slices incubated 4 hr in the dark. Lane 1 contains protein from leaf slices treated with 1 ng/mL victorin; lane 2, protein from leaf slices treated with 1 ng/mL victorin and 1 mM naphthyl acid phosphate (NAP); lane 3, protein from leaf slices treated with 1 mM NAP; and lane 4, protein from leaf slices treated with 5 mM NAP.

(D) Protein gel containing total protein from leaf slices pretreated for 30 min with 100 ng/mL victorin and then rinsed. After another 30 min (i.e., 1 hr after the addition of victorin), the following agents were added: lane 1, 1 mM KCN; lane 2, 10 mM $ZnCl_2$; lane 3, water; and lane 4, 10 mM $LaCl_2$. Leaf slices were then incubated in the dark for an additional 3 hr.

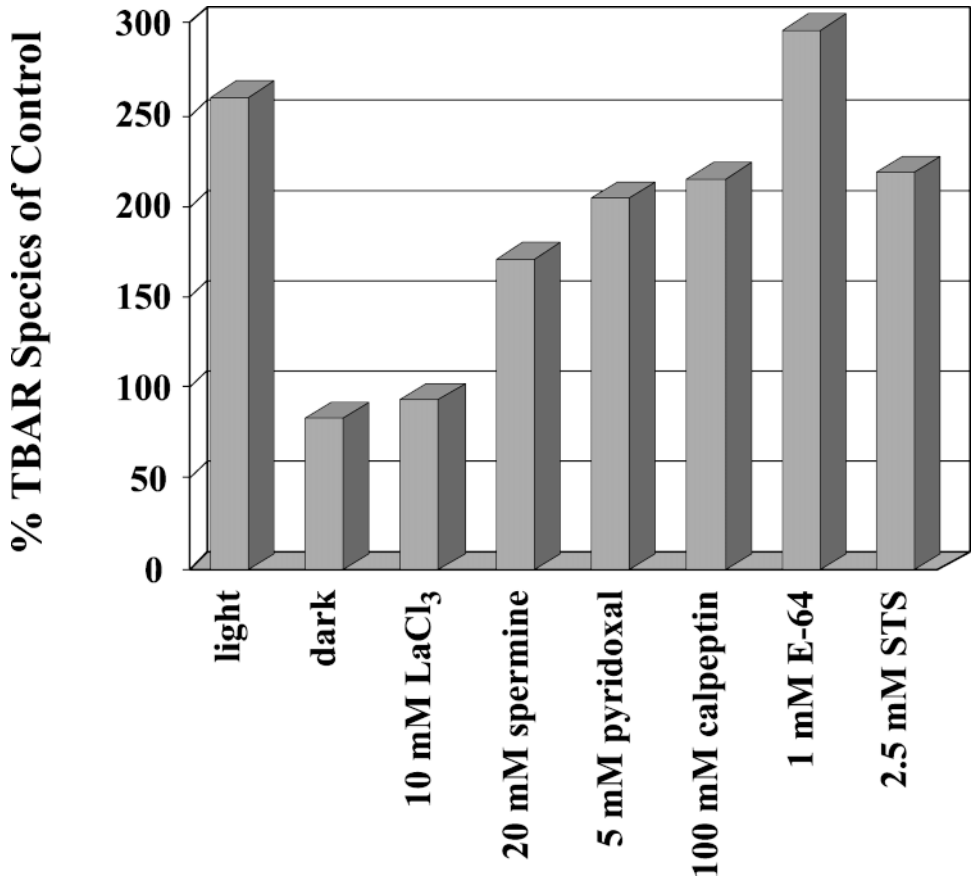

Figure 8. TBARs Generated by Victorin Treatment.

Leaf slices were incubated for 4 hr in the light with the indicated amount of victorin or 100 ng/mL victorin plus the indicated amount of $LaCl_3$, spermine, pyridoxal, calpeptin, E-64, or STS. TBARs were then extracted, quantified, and expressed as a percentage of TBAR accumulation in untreated leaf slices.

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 TBAR detected but did not provide complete protection. Calpeptin was more effective than was E-64 at reducing the amount of TBAR generated (Figure 8).

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 >1 mM prevented LSU cleavage by victorin. Leaf slices treated with 1 mM KCN and victorin showed a minimal amount of LSU cleavage (Figure 9, lane 3). Sodium azide also was effective in preventing LSU cleavage (Figure 9, lane 4), as was dinitrophenol (Figure 9, lane 7), an uncoupler of phosphorylative oxidation. Oligomycin at 5 μg/mL gave partial protection against LSU cleavage (Figure 9, lane 5). Rotenone was ineffective (Figure 9, lane 6) but could be tested only at low concentrations because of solubility limitations.

DISCUSSION

Victorin Induces a Signal Cascade

As more is learned about race-specific elicitors and host-selective toxins, the distinctions between them can become nebulous. For example, a particularly potent elicitor would have the selectivity and lethality of host-selective toxins. Although typically conceptualized as a toxin, victorin clearly induces many plant responses classically associated with

elicitors. The virtually complete protection against victorin by $LaCl_3$ (Figure 5) was particularly interesting for several reasons. $LaCl_3$ protection against victorin indicates that victorin does not directly kill sensitive plants, as might be expected for a necrosis-inducing agent; rather, the plant itself mounts a response to victorin that results in death. Furthermore, the $LaCl_3$ data reveal the presence of a victorin-induced cascade in which calcium acts as a second messenger. Supporting the importance of calcium is the fact that many of victorin's effects, such as stomatal closure and KCl efflux (Ward and Schroeder, 1994), lipoxygenase activity, the respiratory burst (Baggiolini and Wymann, 1990), callose synthesis (Fredrikson and Larsson, 1989), DNA degradation, ethylene responses (Raz and Fluhr, 1992), senescence (Poovaiah, 1987), and phytoalexin synthesis (Stab and Ebel, 1987; Ishihara et al., 1996), have been linked to calcium in other systems.

Ethylene also appears involved in transducing the signal leading to LSU cleavage, because ethylene inhibitors prevented LSU cleavage in leaf slices and provided partial protection against victorin at the whole-leaf level (Figures 5 and 7A). This is particularly interesting given that ethylene also has been implicated in cell death initiated by AAL toxin (Moussatos et al., 1994), an apoptosis-inducing agent (Wang et al., 1996).

Rubisco Cleavage

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 CO_2 (Ferreira and Davies, 1989), and oxidative stress (Casano et al., 1990; Mehta et al., 1992).

Victorin treatment results in a specific cleavage of the Rubisco LSU, 14 amino acids from the N terminus (Figure 3). In the vast majority of plants, this residue is a lysine. In oats (*Avena sativa*), an unpublished nucleotide sequence has indicated that this may be a glutamine residue (GenBank accession number L15300; Duvall et al., 1993). However, this assignment is based on a single nucleotide difference that awaits confirmation.

Victorin-induced LSU cleavage at the 14th amino acid suggests that this site of Rubisco cleavage may be biologically significant as contrasted with in vitro observations in which cleavage at amino acid 14 has been observed repeatedly. Interestingly, lysine-14 often has a post-translational trimethylation modification, as shown by Houtz et al. (1992), who found that lysine-14 contained the modification in eight of 10 plant species examined. The role of the lysine trimethylation is not known, although it has been speculated to protect against proteolytic degradation in the case of a lysine-115 methylation of calmodulin (Gregori et al., 1987).

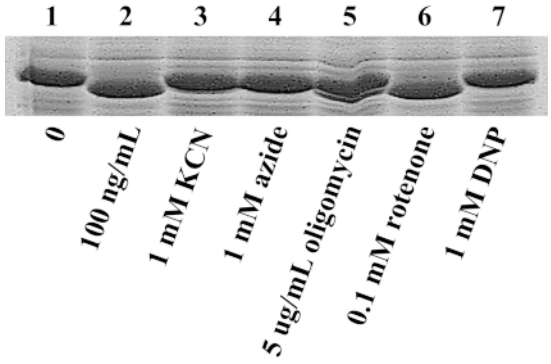

Figure 9. Effect of Respiratory Inhibitors on Victorin-Induced Rubisco Cleavage.

A protein gel containing total protein from leaf slices that were incubated for 4 hr in the dark with the inhibitors indicated. Lane 1 contains protein from leaf slices treated with no victorin; lane 2, protein from leaf slices treated with 100 ng/mL victorin; and lanes 3 to 7, protein from leaf slices treated with 100 ng/mL victorin plus the indicated inhibitors. KCN, potassium cyanide; azide, sodium azide; DNP, dinitrophenol.

Victorin and Chloroplast Function

Our results indicate that victorin impacts chloroplast function, but presumably these effects occur downstream of the initial effect of victorin on the cell. Victorin inhibits light-dependent CO_2 fixation in leaf slices, but this inhibition occurs later than does the inhibition of glycine decarboxylation and is less sensitive to victorin than is glycine decarboxylation (Navarre and Wolpert, 1995). Victorin caused a proteolytic cleavage of Rubisco. Inactivation of Rubisco could account for some of the oxidative damage caused by victorin in light-incubated tissue. However, Rubisco degradation is not the sole source of the oxidative damage observed, because although the protease inhibitor E-64 protects against victorin-induced Rubisco degradation, it does not protect against the accumulation of TBARs.

A role of chloroplasts in the development of victorin symptoms is potentially interesting. If Rubisco is no longer able to use the NADPH produced by photosynthesis, then NADPH may be available for use elsewhere in the cell, including by NADPH oxidases. Furthermore, what is the fate of electrons generated by the photosynthetic apparatus if the Calvin cycle is inoperative or chloroplasts are losing integrity? A disruption in chloroplast electron transport likely would generate large amounts of free radicals in sunlight. Such a mechanism could initiate lipid peroxidation and is consistent with TBARs being detected in victorin-treated leaf slices incubated in the light but not in the dark. Furthermore, among all organisms, O_2 concentration is the highest

in plants and may occur in the chloroplast at over 300 μM, compared with 0.1 μM near mammalian mitochondria (Robinson, 1988; Scandalios, 1993). Given the chloroplast's light-harvesting apparatus and the high oxygen concentrations, plants have a mechanism to produce large quantities of reactive oxygen species via a mechanism distinct from other organisms. Thus, any perturbation by victorin of chloroplast function has the potential to produce significant amounts of free radicals.

In this context, a possible role for chloroplasts in hypersensitive cell death observed in response to pathogens is interesting. Purohit and Tregunna (1975) found that 1% CO_2 inhibited lesion production in tobacco plants inoculated with two viral pathogens, and they speculated about a photorespiratory link. Light is required for the hypersensitive response in some diseases of tomato (Peever and Higgins, 1989) and rice (Guo et al., 1993). Light is required also 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 host-selective toxin, has a mitochondrial site of action yet causes rapid chlorophyll loss in the light but not in the dark (Bhullar et al., 1975).

Parallels between Victorin Effects and Senescence

Victorin appears to trigger senescence, a form of programmed cell death. Furthermore, many of the effects of victorin, including DNA laddering, mitochondrial dysfunction, lipid peroxidation, ordered proteolytic events, and calcium and phosphatase involvement, are also characteristic of mammalian programmed cell death. Symptoms of victorin treatment resemble an accelerated senescence, particularly when using lower victorin concentrations that give symptoms time to develop. Rubisco degradation is a hallmark of senescence, as is cholorophyll loss. In high humidity and at low victorin concentrations, the most noticeable symptom of treatment with victorin is a near-total loss of chlorophyll and the plant remains turgid (D.A. Navarre and T.J. Wolpert, unpublished data). Lipid peroxidation (Figure 8) and ethylene evolution occur during senescence and also occur in victorin-treated tissue. Furthermore, ethylene inhibitors prevented Rubisco cleavage by victorin.

Victorin-treated leaves have the pronounced DNA ladder characteristic of apoptosis—a word that refers to leaf senescence (Figure 4). Polyamines are known to prevent or delay senescence, and spermine was effective at preventing LSU cleavage (Figure 7A). CO_2 is known to delay senescence, and elevated CO_2 concentrations also delayed the effect of victorin (D.A. Navarre and T.J. Wolpert, unpublished data) on whole plants and detached leaves, and bicarbonate prevents LSU cleavage (Figure 7B). Victorin-induced cleavage of Rubisco appears to be mediated by a cysteine protease, and several cloned senescence-associated genes show sequence similarity to cysteine proteases (Hensel et al., 1993; Lohman et al., 1994).

In view of victorin's inhibition of the GDC, linkages between senescence and photorespiration in the literature are particularly interesting. Barley and Arabidopsis glycine decarboxylase mutants undergo premature senescence (Somerville and Ogren, 1982; Blackwell et al., 1990). Widholm and Ogren (1969) showed that C_3 plants in photorespiratory conditions underwent premature senescence, whereas C_4 plants did not. Other studies also suggest a link between photorespiration and senescence (Martin and Thimann, 1972; Mondal and Choudhuri, 1982; Satler and Thimann, 1983).

That photorespiratory stress is involved in some instances of senescence is not surprising because oxidative stress is generally associated with the induction of senescence. Mehta et al. (1992) suggested that the cellular and stromal oxidative state initiate senescence. Thimann and Satler (1979) found that treatments that caused oat stomates to close also accelerated senescence. Stomatal closure in sunlight can lead to superoxide production and photoinhibition and photoxidative damage (Scandalios, 1993). Victorin causes stomates to close within 30 min of application (D.A. Navarre and T.J. Wolpert, unpublished data), and this places the plant under even greater oxidative stress in the light, because stomatal closure decreases the intracellular CO_2 concentration, thus increasing photorespiration. Thus, victorin not only causes sensitive plants to increase O_2 fixation (by closing stomates) but also prevents the plant from coping with increased photorespiration by inhibiting glycine decarboxylation.

What is a possible role of an accelerated senescence response in a host–pathogen interaction? It might be to a plant's benefit to enter senescence prematurely and to sacrifice part of its biomass, while recycling nutrients from this senescing tissue and at the same time depriving the pathogen of living cells and sustenance. Conversely, the pathogen might benefit by causing the host tissue to self-destruct, thereby negating host defenses in those cells and at the same time freeing up a rich pool of nutrients. Such a strategy might be to the plant's advantage with biotrophic pathogens but to the plant's disadvantage with necrotrophic pathogens.

Victorin and Glycine Decarboxylation

Is the binding of victorin by the GDC the initial step in the cascade described in this study? If the binding of victorin by the GDC is the crucial event, how does inhibiting the GDC lead to all of the secondary effects described? We do know that victorin binds to two components of the GDC and that victorin is a potent inhibitor of the GDC. It is also known that GDC mutants undergo early senescence. Thus, in the mutants, the mitochondrial GDC inactivity leads to chloroplastic and cell-wide events. Like victorin, T-toxin is a host-selective toxin but is known to have a mitochondrial site of action, yet it also inhibits photosynthesis and causes stomatal closure (Arntzen et al., 1973). CO_2 lessens the toxicity of victorin, which would seem to implicate the GDC. Furthermore, pyridoxal prevents cleavage of the LSU by victorin, and this is

significant because pyridoxal phosphate competes with binding of victorin by the P protein of victorin (T.J. Wolpert, unpublished data). Finally, the mitochondrion is known to play a pivotal role in both mammalian senescence and apoptosis (Mignotte and Vayssiere, 1998; Rosse et al., 1998). Thus, there is a precedence for an initial mitochondrial event leading to a cell-wide cascade of effects. However, it is possible that victorin directly interacts with some other cellular component in addition to the GDC and that it is this interaction that is responsible for some of victorin's effects.

METHODS

Plants and Materials

Oat (*Avena sativa*) seedlings were grown in a growth chamber for 5 to 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 ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) cleavage, 10 leaf slices of ~2 mm were incubated in a final volume of 100 μL 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 μL of water-saturated phenol and a small amount of sand were added. Leaf slices were then homogenized in microcentrifuge tubes, after which 200 μL of homogenization buffer (50 mM Tris, 0.7 M sucrose, 5 mM DTT, 100 mM KCl, and 5 mM EDTA, pH 7.0) was added, and the two phases were mixed. Samples were centrifuged in a microcentrifuge for 2 min at 12,000 rpm, and the phenol phase was collected. One milliliter of 0.1 M ammonium acetate in methanol was added to the phenol phase, and proteins were precipitated for at least 2 hr at −20°C. Samples were centrifuged for 5 min at 12,000 rpm in a microcentrifuge, and the resulting pellet was rinsed once with methanol. The pellet was resuspended in 100 μL of sample buffer (6 M urea, 2% [w/v] SDS, 60 mM Tris, and 20 mM DTT, pH 6.8), and typically 10 to 20 μL of this sample was loaded onto a 14% SDS–polyacrylamide gel. To check whether a cleaved large subunit (LSU) product was associated with thylakoid membranes, leaf slices were treated for 4 hr 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 phenylmethylsulfonyl fluoride, and 0.1 mM E-64. The homogenate was centrifuged for 15 min at 12,000 rpm in a microcentrifuge. The resulting pellet was considered to be the thylakoid fraction and the supernatant the stromal fraction.

Detection of Lipid Peroxidation

Thiobarbituric acid–reactive species (TBARs), of which malondialdehyde is expected to be the primary form, were assayed essentially as described by Dhindsa et al. (1981). Twenty leaf slices were incubated in either the dark or light (15,000 lux) for the time and treatments indicated. The average of three replications per treatment from one representative experiment is shown.

Purification of Rubisco

Thin (~1 mm) leaf slices from 20 blades of 7-day-old plants were incubated in the dark at 25°C for 4 hr with 100 ng/mL victorin. The leaf slices were rinsed with water and homogenized in 20 mL of 20 mM Tris–HCl, 5 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM E-64, and 0.1 mM leupeptin, pH 8.0, with a chilled mortar and pestle. The homogenate was filtered through two layers of cheesecloth and centrifuged at 5000 rpm for 5 min. The supernatant was collected and centrifuged at 60,000*g* for 60 min at 4°C. The resulting pellet was discarded, and the supernatant was loaded directly onto a C_4 HPLC column (Vydac; Phenomenex, Torrance, CA) equilibrated in buffer A (2% MeOH and 0.2% trifluoroacetic acid). Protein was then eluted with a linear gradient of 0 to 70% acetonitrile in buffer A. Consecutive fractions were analyzed by SDS-PAGE. The purified Rubisco fraction was then electrophoresed on a 14% SDS–polyacrylamide gel, and the gel was electroblotted onto an Immobilon-P membrane (Millipore Corporation, Bedford, MA) as previously described (Navarre and Wolpert, 1995). The N-terminal amino acid sequence was determined on a gas-phase protein sequencer (model 475A; Applied Biosystems, Foster City, CA) operated by the Central Service Laboratory 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 to 6 hr. Approximately 10 leaf segments (10 cm) were used per treatment and incubated in the dark at room temperature with slow shaking. Treated leaves were frozen in liquid nitrogen, and the cetyltrimethylammonium bromide (CTAB) procedure (Richards et al., 1994) was used to extract DNA. DNA was separated on a 2% agarose gel, stained with ethidium bromide, and photographed with a Gelprint 2000i imaging system (BioPhotronics, Ann Arbor, MI).

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