Identification of the 100-kD Victorin Binding Protein from Oats

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The fungus Cochliobolus victoriae, the causal agent of victoria blight of oats, produces the host-specific toxin victorin. Sensitivity of oats to victorin, and thus susceptibility to the fungus, is controlled by a single dominant gene. This gene is believed to also confer resistance to the crown rust pathogen Puccinia coronata. In the case of victoria blight, the gene has been hypothesized to condition susceptibility by encoding a toxin receptor. A 100-kD victorin binding protein (VBP) has been identified; it binds radiolabeled victorin derivatives in a ligand-specific manner and in a genotype-specific manner in vivo. The VBP may function as a toxin receptor. In vitro translation coupled with indirect immunoprecipitation was used to identify the mRNA for the 100-kD VBP, and fractionated mRNAs were used to prepare cDNA libraries enriched in the relative abundance of cDNA for the 100-kD VBP. A 3.4-kb cDNA clone was isolated that, when subjected to a 400-bp 5’ deletion, was capable of directing the synthesis of a protein in Escherichia coli, which reacted to an antibody specific for the 100-kD VBP. Peptide mapping, by limited proteolysis, indicated that the protein directed by the cDNA is the 100-kD VBP. Nucleotide sequence analysis of the cDNA revealed extensive homology to a previously cloned cDNA for the P protein component of the multienzyme complex glycine decarboxylase. Glycine decarboxylase is a nuclear-encoded, mitochondrial enzyme complex. Protein gel blot analysis indicated that the 100-kD VBP copurifies with mitochondria. Based on analysis of in vitro translation products, nucleotide sequence homology, mitochondrial localization, and the widespread species distribution of the 100-kD VBP, we concluded that the 100-kD VBP is the P protein component of glycine decarboxylase.

INTRODUCTION

The fungus Cochliobolus victoriae, causal agent of victoria blight of oat, is pathogenic because it produces the host-specific toxin victorin. Sensitivity to victorin, and thus susceptibility to toxin-producing isolates of the fungus, is conditioned by the dominant allele at the Vb locus (Scheffer, 1976). Genotypes of oats homozygous recessive at the Vb locus (vb vb) are neither sensitive to the toxin nor susceptible to the pathogen. Victoria blight arose as a new disease of oats during the early 1940s as a consequence of the widespread introduction of oat cultivars carrying the Pc-2 gene for resistance to Puccinia coronata, causal agent of crown rust of oats (Meehan and Murphy, 1946, 1947). Attempts to genetically resolve toxin sensitivity from rust resistance have failed (Rines and Luke, 1985). These results have led to the assumption that the Pc-2 gene and the Vb gene are the same. Thus, identification of the gene that conditions toxin sensitivity may simultaneously lead to the identification of a rust resistance gene.

The host-specific toxin victorin has been characterized as a group of closely related cyclized pentapeptides (Macko et al., 1985; Wolpert et al., 1985, 1986). Victorin C, the most prevalent form of the toxin found in culture filtrates of C. victoriae, was used to prepare biologically active, labeled derivatives (Wolpert et al., 1988; Wolpert and Macko, 1989), and an 125I-labeled derivative of victorin C was used to identify a 100-kD victorin binding protein (VBP). The 100-kD VBP binds radiolabeled victorin in a ligand-specific manner and, in vivo, binds victorin only in oats that carry the dominant allele at the Vb locus (Wolpert and Macko, 1989). Binding analysis also indicated ligand-specific binding of 125I-labeled victorin in vivo to a 15-kD VBP in both susceptible and resistant oats (Wolpert and Macko, 1989). The observation that binding to the 100-kD VBP in vivo is specific to susceptible genotypes suggests that the interaction of victorin with the 100-kD VBP is related to the genotype-specific response of oats to victorin.

Our current hypothesis is that the 100-kD VBP is the site of action of the toxin or is associated with the site of action and, as such, may be the product of the Vb locus. Our objective

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is to evaluate this hypothesis. This article reports the molecular cloning and expression in *Escherichia coli* of a cDNA for the 100-kD VBP, identification of the protein by nucleotide sequence analysis of the cDNA, and biochemical verification of the identity of the 100-kD VBP.

**RESULTS**

**In Vitro Translation and mRNA Enrichment**

In vitro translation of isolated poly(A)^+ RNA (mRNA) coupled with indirect immunoprecipitation was used for monitoring the enrichment of the relative abundance of the mRNA for the 100-kD VBP. The mRNA encoding the 100-kD VBP was present in relatively low abundance in total mRNA fractions (Figures 1A and 1B, lanes T). However, mRNA from the microsomal fraction (Figures 1A and 1B, lanes M) was substantially enriched for the mRNA for the 100-kD VBP. Further enrichment was achieved by size fractionation of mRNA, as indicated by in vitro translations, of the upper fractions of 5 to 20% denaturing sucrose gradients of membrane-bound mRNA (Figures 1A and 1B, lanes F). The isolation of size-fractionated, membrane-bound mRNA enriched the presence of the 100-kD VBP mRNA by ~50- to 100-fold (Figure 1). Total translation products (Figure 1A) indicated the presence of high molecular mass protein products in all fractions of mRNA analyzed; this suggested that the apparent enrichment for the mRNA for the 100-kD VBP was not an artifact of protection against RNase degradation. Comparison of total translation products from membrane-bound mRNA (Figure 1A, lane M) and size-fractionated, membrane-bound mRNA (Figure 1A, lane F) showed an enrichment for high molecular mass protein products, confirming that centrifugation on sucrose gradients size fractionated the mRNA. Also, comparisons of total translation products (Figure 1A) with immunoprecipitated products (Figure 1B) showed that antibody preparations were specific to the 100-kD VBP. The immunoprecipitated, in vitro translation product (Figure 1B) migrated with an apparent mass of ~111 kD on SDS-polyacrylamide gels when compared to protein standards. This is ~5 kD larger than the mass indicated by SDS-polyacrylamide gel electrophoresis of the 100-kD VBP identified by in vivo and in vitro victorin binding (Wolpert and Macko, 1989), and suggested that the VBP may exist as a precursor that is proteolytically processed to the mature form of the VBP.

**cDNA Cloning**

Enriched mRNA preparations, derived from membrane-bound, size-fractionated mRNA, were used to produce a λ gt11 cDNA library. Based on estimates by densitometric scanning of in vitro translation products (data not shown), the relative abundance of the mRNA for the 100-kD VBP in the membrane-bound, size-fractionated mRNA preparations was ~1.0%. Assuming no bias in the recovery of cloned 100-kD VBP cDNAs and that one of six randomly cloned cDNAs is in the proper orientation for expression, the frequency of antibody-positive recombinants was expected to be ~0.17%. Screening of the cDNA library with the antibody to the 100-kD VBP resulted in the isolation of antibody-positive recombinants at the frequency of 0.16%, which is very close to the expected frequency. Analysis of the insert cDNAs from these recombinants consistently identified two types, one with a 0.8-kb insert and one with a 1.2-kb insert.

An RNA gel blot was prepared from total oat leaf RNA and probed with the 1.2-kb cDNA clone that directed an antibody-positive reaction. Results indicated that the cDNA had been derived from an mRNA of sufficient size to encode the 100-kD VBP. The 1.2-kb cDNA hybridized to an mRNA of 3.4 to 4.0 kb compared to RNA standards (Figure 2). Thus, the following observations indicated that the 1.2-kb cDNA was a partial cDNA to the 100-kD VBP: (1) the antibody preparation was specific (Wolpert and Macko, 1991); (2) the antibody screen consistently selected the same recombinants and at the expected frequency; (3) the induced cDNAs resulted in an antibody-positive reaction; and (4) the cDNA was derived from an mRNA of the appropriate size.
To obtain a full-length cDNA, a library was prepared from size-fractionated cDNA and screened with antibody to the 100-kD VBP. Antibody-positive recombinants were not detected and, thus, recombinants were screened with $^{32}$P-labeled probe prepared from the 1.2-kb cDNA previously isolated. This approach led to the isolation of a 3.4-kb cDNA clone.

**Verification of the Identity of the cDNA clone**

Confirmation of the identity of the 3.4-kb cDNA clone required a comparison of the 100-kD VBP from oats and the protein expressed from the cDNA. Production of an expressible construct of the 3.4-kb cDNA was attempted by ligation in all three reading frames and in both orientations. However, these constructs did not yield antibody-positive recombinants. Based on these results, the possibility of proteolytic processing and the fact that the 100-kD VBP from oats has a mass of $\sim$106 kD and, thus, should require an $\sim$3-kb reading frame, we concluded that the cDNA might possess a 5' leader sequence that interfered with expression in *E. coli*. Therefore, 5' deletions of the cDNA were prepared. The approach indicated that the minimal 5' deletion resulting in high-level expression was $\sim$0.4 kb. A selected recombinant, induced by isopropyl β-D-thiogalactopyranoside (IPTG), was capable of directing the synthesis of an $\sim$103-kD protein that was antibody-positive when screened with polyclonal antibodies raised against the 100-kD VBP (Figure 3). Protein expressed in *E. coli* from the 5'-deleted cDNA was isolated and compared with the 100-kD VBP from oats.

Direct structural comparison of the proteins was conducted with the Cleveland method for comparative peptide mapping (Flannery et al., 1989). The peptide fragments generated by limited proteolysis of the 100-kD VBP from oats and the cDNA construct expressed in *E. coli* were detected by immunoblotting. The results of the comparison indicated that the peptide pattern resulting from partial proteolysis with each of three different proteases, *Staphylococcus aureus* V8 protease, subtilisin, and chymotrypsin, was virtually identical for the protein from oats and the cDNA expressed in *E. coli* (Figure 4). A quantitative method has been developed for calculating the degree of similarity between proteins generated by the Cleveland peptide mapping procedure (Calvert and Gratzer, 1978). Based on this method and incorporating the data from all three proteases, the probability that the extent of matching observed between the 100-kD VBP from oats and the protein from *E. coli* expressing the cDNA clone occurred by chance is $\sim 1 \times 10^{-17}$. Thus, comparative peptide mapping indicated that a full-length cDNA clone of the 100-kD VBP had been isolated.

**Figure 2.** Gel Blot Analysis of Total Oat Leaf RNA.

Ten micrograms of total RNA was isolated from the leaves of susceptible oats. A 1.2-kb cDNA, which conferred an antibody-positive response in λ gt11, was used as the probe. Markers at left indicate the molecular lengths of RNA standards in kilobases.

**Figure 3.** Expression of the 5'-Deleted cDNA in *E. coli*.

Immunoblot analysis was conducted with the anti-100-kD-VBP antibody on total protein extracts of *E. coli*, lanes 1 to 4, and oat leaves, lane 5. Lane 1 was loaded with a sample from bacteria transformed with pTTQ19 and no IPTG induction; lane 2 is the same as lane 1 except that bacterial cells were induced with 10 mM IPTG; lane 3 was loaded with a sample from bacteria transformed with the $\sim$400-bp deleted cDNA and no IPTG induction; and lane 4 is the same as lane 3 except that bacterial cells were induced with 10 mM IPTG. Markers at left indicate the migration of protein standards with masses given in kilodaltons.
Figure 4. Comparison of the Proteolysis Products of the 100-kD VBP and the Protein Expressed from the 5'-Deleted cDNA in E. coli.

Immunoblot analyses conducted with the anti-100-kD VBP of partial proteolysis products of protein from oat leaf tissue, lanes 1, 3, and 5, or transformed E. coli, lanes 2, 4, and 6, are shown. E. coli was transformed with pUC19 containing the 5'-deleted, full-length cDNA for the 100-kD VBP. Lanes 1 and 2 show samples subjected to 100 ng of S. aureus V8 protease; lanes 3 and 4 show samples subjected to 10 ng of subtilisin; and lanes 5 and 6 show samples subjected to 3 μg of chymotrypsin. All protease incubations were performed in the stacking gel at room temperature for 30 min.

cDNA Sequence Analysis

DNA sequence analysis of the 3427-bp cDNA for the VBP revealed a 3096-bp open reading frame encoding a protein containing 1032 amino acid residues (GenBank accession number U11693). The first possible translation initiation site is located at nucleotide 71 and is in frame with the largest open reading frame and all other possible initiation sites contained in the first 1292 bp of the cDNA. Sequence data indicated a 70-bp 5' untranslated region and a 241-bp 3' untranslated region downstream from a termination codon beginning at nucleotide 3167. There was no AATAAA polyadenylation signal in the 3' region. However, it is likely that the sequence AATACA beginning at position 3385, 18 bp upstream of the polyadenylation site, serves as the signal for polyadenylation.

Data base searches identified a cDNA sequence from pea that showed 74% nucleotide identity in a 2684-bp overlap with the cDNA for the 100-kD VBP. A comparison of the deduced amino acid sequence of the 100-kD VBP cDNA and the pea cDNA indicated 83% amino acid identity (Figure 5). The pea cDNA codes for the P protein component of the glycine decarboxylase multienzyme complex (Turner et al., 1992). The P protein is localized in the mitochondria and is presumed to have a mitochondrial targeting sequence. N-terminal amino acid sequence analysis of the mature P protein from peas has indicated that cleavage of the targeting sequence occurs between serine-86 and isoleucine-87. Comparison of the deduced amino acid sequence of the P protein and 100-kD VBP demonstrated identity in 15 of the first 17 amino acids of the inferred translation initiation site for both cDNAs (Figure 5). By analogy to the pea sequence, the deduced amino acid sequence starting at the inferred initiation site and extending to the valine residue at amino acid 60 comprises the mitochondrial targeting sequence for the 100-kD VBP (Figure 5). The inferred mitochondrial targeting sequence of the 100-kD VBP constitutes the region of greatest dissimilarity in the comparison of the deduced amino acid sequence of the pea P protein and the 100-kD VBP. The targeting sequence for the protein from peas has been inferred to be 86 amino acid residues, whereas the analogous sequence in oats is 59 residues. Comparison of the inferred mature forms of the proteins indicates 91% amino acid similarity between oats and peas. The inferred mature form of the 100-kD VBP also displays 71% amino acid similarity to the P protein of E. coli (GenBank accession number L20872), humans, and chickens (Kume et al., 1991).

Genotype Distribution and Localization of the VBP

Prior to sequence analysis of the cDNA for the 100-kD VBP, we conducted protein gel blot analysis and determined that a protein immunologically cross-reactive to the 100-kD VBP was present in all plant species and organisms tested (Figure 6). Equivalent results were obtained by in vitro binding of 125I-labeled victorin and gel blot analysis of genomic DNA when probed with the cDNA for the VBP (data not shown). These results showed that a protein homologous to the 100-kD VBP existed in samples as diverse as cultured E. coli cells and ovine liver tissue (Figure 6) and are consistent with results obtained by Loschke et al. (1994).

The glycine decarboxylase complex is found in animals, plants, and prokaryotes and is located in the mitochondria of eukaryotic organisms (Kume et al., 1991). Protein gel blot analysis conducted with antibody to the 100-kD VBP on a constant amount of protein from a total protein fraction, a 10,000g for 10-min pellet, and mitochondria purified by Percoll gradient centrifugation indicated a progressive enrichment for the relative abundance of the 100-kD VBP (Figure 7). Similar results were obtained when fractions were analyzed by in vitro binding of 125I-labeled victorin (data not shown). These results indicated that the 100-kD VBP is compartmentalized in the mitochondria of oats.

DISCUSSION

cDNA clones were selected, either directly or indirectly, by their immunoreactivity to antibody to the 100-kD VBP. Antibody
preparations appeared specific to the 100-kD VBP based on immunoprecipitations of in vitro translation products from oat leaf mRNA (Figure 1). Also, antibody preparations had previously been shown to be specific to the 100-kD VBP when used for indirect immunoprecipitations of in vivo and in vitro victorin-labeled proteins and gel blot analysis of total protein extracted from oat leaf tissue (Wolpert and Macko, 1991). Confirmation that the 3.4-kb cDNA clone isolated was a clone for the 100-kD VBP was established by Cleveland mapping, which provided a direct structural comparison of the 100-kD VBP from oat and the protein resulting from expression of the cDNA in E. coli (Figure 4).

Antibody was also used to identify proteins homologous to the 100-kD VBP in species other than oats. Gel blot analysis indicated that all plants tested contained a 100-kD protein that reacted with antibody to the 100-kD VBP (Figure 7). A 100-kD protein homologous to the VBP was detected in organisms as diverse as E. coli and bovine. The apparent widespread distribution of protein homology suggests a common function for the 100-kD VBP, such as glycine decarboxylation. Furthermore, consistent with the observation that high concentrations of glycine decarboxylase and thus the P protein are restricted to the bundle sheath cells of C₃ plants (Hylton et al., 1988) is the fact that the quantity of the 100-kD VBP appeared lower in sorghum and maize than in the other plants tested, which were all C₄ (Figure 7).

Nucleic acid sequence analysis of the cDNA for the 100-kD VBP established extensive homology with a cDNA clone from pea for the P protein component of the multienzyme complex glycine decarboxylase; we found 91% amino acid similarity between the mature forms of the two proteins (Figure 4). Glycine decarboxylase is found in prokaryotes and eukaryotes and in the latter is localized in the mitochondria. Gel blot analysis of subcellular protein fractions indicated that the relative abundance of the 100-kD VBP was greatly increased in purified mitochondria (Figure 7). This finding provides supporting evidence for the identification of the 100-kD VBP as the P protein component of glycine decarboxylase.

The presence of a precursor form of the protein, suggested by different electrophoretic mobilities relative to protein standards, of the 100-kD VBP from in vitro translations and iodine-125 labeling is also consistent with the identification of

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**Figure 5. Comparison of the Deduced Amino Acid Sequence of the cDNA Clones from Oat and Pea.**

The inferred amino acid sequences are indicated using single-letter codes, and regions where gaps exist are indicated by horizontal dots. The top line is the sequence deduced from the cDNA for the 100-kD VBP isolated from oat, and the bottom line is the sequence deduced for the P protein from pea. Identity between amino acid comparisons is indicated by a solid vertical bar and similarity by one or two dots. Two dots indicate a greater degree of similarity than one dot. P protein data are from Turner et al. (1992).
Figure 6. Species Distribution of the 100-kD VBP.

Immunoblot analyses conducted with the anti-VBP antibody of protein extracts from spinach (Sp), Arabidopsis (A), pea (P), tomato (T), wheat (W), sorghum (S), maize (O), Chlorella (Ch), bovine liver (L), and E. coli (E) are shown. The marker at left indicates the migration of the 100-kD VBP.

the 100-kD VBP as the P protein component of glycine decarboxylase. The P protein possesses an N-terminal mitochondrial import signal that is proteolytically cleaved upon mitochondrial import. The apparent association of the mRNA of the 100-kD VBP with a 30,000g particulate fraction (membrane-bound) suggests that the 100-kD VBP is cotranslationally imported into mitochondria. Cotranslational import has been observed for the majority of yeast mitochondrial proteins (Fujiki and Verner, 1991).

An analysis of the 100-kD VBP was motivated by the observations that binding of $^{125}$I-labeled victorin appears to be ligand specific and, in vivo, genotype specific (Wolpert and Macko, 1989). Ligand-specific binding to the 100-kD VBP demonstrates that the interaction of victorin with the protein is nonrandom and indicates structural specificity. The observation that binding is genotype specific suggests that the interaction of victorin with the 100-kD protein is involved in the genotype-specific response of susceptible oats to victorin. A description of genotype-specific binding was initially achieved with $^{125}$I-labeled victorin. More recently, we have found that biotinylated toxin, detected by anti-biotin antibody, selectively labeled intact mitochondria of susceptible and not resistant oats (H. Israel, T.J. Wolpert, and V. Macko, unpublished data). Thus, the genotype specificity of the interaction of victorin with the 100-kD VBP has been indicated by two independent approaches.

Akimitsu et al. (1992) have also identified a 100-kD VBP that was detected with anti-victorin antibody. This procedure identified immunoreactive bands that migrated with apparent masses of 100, 65, and 45 kD. Because of the variability of binding to the 65- and 45-kD bands, the authors suggested that these bands are proteolytically derived from the 100-kD protein. Ligand-specific binding of victorin to the 100-kD protein could not be assessed, and the approach did not detect genotype-specific binding by the 100-kD protein. Although it is possible that a different 100-kD VBP has been identified, it is probable that the use of the anti-victorin antibody detected the same 100-kD protein as $^{125}$I-labeled victorin. It is likely that genotype-specific binding was not detected in vivo with the anti-victorin antibody, because the binding observed was a result of in vitro binding that occurred as a consequence of homogenizing the tissue in a nondenaturing buffer (Akimitsu et al., 1992). Akimitsu et al. (1992) stated that they detected less binding to X424 (resistant) but not to other resistant cultivars, thus suggesting an explanation for the genotype-specific binding observed with $^{125}$I-binding comparisons between X469 (susceptible) and X424 (resistant) (Wolpert and Macko, 1989). However, a difference in the amount of 100-kD VBP between X469 (susceptible) and X424 (resistant) has not been detected with an antibody to the 100-kD VBP either by gel blot analysis of total protein or indirect immunoprecipitation of in vitro-labeled protein (Wolpert and Macko, 1991) or by direct analysis of in vitro binding (Wolpert and Macko, 1989). Furthermore, this explanation fails to account for the fact that in vivo genotype-specific binding was also detected in binding comparisons of cultivars Park (susceptible) and Rodney (resistant) (Wolpert and Macko, 1989).

Akimitsu et al. (1993) described an attempt at the subcellular localization of the 100-kD VBP. Based on marker enzyme analysis, they reported that the 100-kD VBP cosegregated with mitochondria and plasma membrane fractions in continuous sucrose gradient analysis of extracts from dark-grown seedlings. This finding is consistent with our findings, which

Figure 7. Identification of the 100-kD VBP in Oat Cell Fractions.

Immunoblot analyses conducted with the anti-VBP antibody of protein fractions from oat X469 are shown. Lane T contains 2 $\mu$g of total protein; lane P, 2 $\mu$g of protein from the pellet resulting from centrifugation for 10 min at 10,000g; and lane M, 2 $\mu$g of protein from purified mitochondria. Markers at left indicate molecular mass standards in kilodaltons.
indicated that the 100-kD VBP is a component of the mitochondrial enzyme glycine decarboxylase. However, they did not detect 100-kD VBP in purified mitochondria (or plasma membrane) and concluded that the 100-kD VBP is not located in the mitochondria (or plasma membrane). An indication of the integrity of their purified mitochondria was not included. It is possible that the purified mitochondria used in their study had damaged membranes, which could result in loss of the 100-kD VBP.

Photorespiratory glycine arises as a consequence of the interaction of O2 with the enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase. In green tissue, glycine decarboxylase has a major role in the recovery of carbon and energy lost due to photorespiration, and mutations in the photorespiratory cycle are lethal to plants (Artus et al., 1986; Husic et al., 1987). Thus, if the sole effect of toxin interaction with the P protein is the inhibition of glycine decarboxylase activity, this would apparently be sufficient to cause cell death in green tissue. Photorespiration, by definition, only occurs in green tissues. However, victorin is believed to affect non-green or dark-grown tissues. The synthesis of glycine decarboxylase and the P protein component of the complex (Kim et al., 1991; Turner et al., 1992) is light induced in green tissues, but the complex is also present in non-green and dark-grown tissues (Walker and Oliver, 1986) and in non-plants. The 100-kD protein is present in dark-grown oat tissue as indicated by the cosegregation of the 100-kD VBP with mitochondria in continuous sucrose gradients of homogenates of etiolated oat tissue (Akimitsu et al., 1993). Because glycine decarboxylase in plants has been studied primarily in the context of photorespiration, it is not clear whether binding of victorin to the P protein would be sufficient to cause cell death in etiolated or non-green tissues. However, it may be significant to note that in humans, deficiency in glycine decarboxylase activity leads to hyperglycinemia, a rapidly fatal disease (Kume et al., 1988).

Our current interpretation of the 125I-labeled victorin binding data is that the 100-kD VBP is the site of action and/or critical to the activity of victorin, but may or may not be the product of the Vb gene (Wolpert and Macko, 1989, 1991). The isolation of a cDNA clone for the 100-kD VBP provides the technology for tracking the gene for the 100-kD VBP in segregating lines of oats. This could, in turn, provide a genetic answer to whether the gene for the 100-kD VBP cosegregates with the phenotype of toxin sensitivity (and consequently susceptibility to the fungus) and, thus, whether it is the likely product of the Vb gene and the rust resistance Pc-2 gene. It is interesting to note that photorespiration is an obligatory process in plants grown in the presence of oxygen, and inhibition of glycine decarboxylase would lead to an accumulation of glycine (Husic et al., 1987). Although complete inhibition of glycine decarboxylase in leaf tissue would inevitably be lethal, partial or gradual inhibition of the enzyme complex may serve to provide a pathogen with an abundant and obligatory source of carbon and nitrogen. The identification of the 100-kD VBP as the P protein component of glycine decarboxylase suggests that victorin, or a victorin-like molecule, may function as a pathogenic determinant in ways other than toxicity.

Based on (1) the widespread species distribution of the 100-kD VBP, suggesting a common and basic metabolic function of the 100-kD VBP; (2) in vitro translation analysis, suggesting a precursor form of the 100-kD VBP that is proteolytically processed; (3) nucleotide sequence homology of the cDNA for the 100-kD VBP with the P protein component of glycine decarboxylase; and (4) the mitochondrial localization of the 100-kD VBP, we concluded that the 100-kD VBP is the P protein component of glycine decarboxylase. The identification of the 100-kD VBP as the P protein component of the glycine decarboxylase multienzyme complex provides a clear biochemical target for investigations of the mode of action of victorin and the biochemical basis for selectivity in genotypes sensitive to the toxin. We are currently pursuing both a biochemical and a genetic analysis of the role of the P protein in victoria blight of oats.

METHODS

Plant Material

Oat plants used for RNA, mitochondria, and protein isolations were X469 (Frey et al., 1971a, 1971b), which is homozygous for the Vb allele (susceptible). The plants grown for analysis of the presence of a protein homologous to the 100-kD victorin binding protein (VBP) were maize, tomato, pea, Arabidopsis ecotype Columbia, sorghum, and wheat. Spinach was obtained from a local supermarket. All other plants were grown in a growth chamber under a 16-hr photoperiod (23°C light, 20°C dark, 60% relative humidity) for 7 to 10 days. Cultured Chlorella strain N1a cells were the kind gift of R. Meints (Oregon State University, Corvallis, OR).

RNA Isolation

Total RNA was isolated by phenol-chloroform extraction and LiCl precipitation (Reddy and Gilman, 1990). Poly(A)⁺ RNA (mRNA) was purified by oligo(dT) cellulose chromatography (Kingston, 1990). Membrane-bound mRNA was prepared by grinding leaf tissue in liquid nitrogen and suspending the powdered tissue (1 g fresh weight per 4.12 mL of buffer) in buffer containing 0.25 M Tris, 35 mM MgCl₂, 5 mM DTT, 0.2 M sucrose, 25 mM EDTA, 80 mM KCl, and 80 g/mL cycloheximide, pH 8.5. The homogenate was passed through four layers of cheesecloth and then centrifuged at 20000g for 5 min. The supernatant was then centrifuged at 30,000g for 10 min, and the resulting pellet was resuspended in buffer consisting of 0.18 M Tris, 0.05 M LiCl, 4.5 mM EDTA, 1% SDS, pH 8.2. This extract was then passed through phenol-chloroform and subjected to oligo(dT) cellulose chromatography (Kingston, 1990). Membrane-bound mRNA was size fractionated by sucrose density gradient centrifugation according to the methods of Bishop et al. (1969).

Briefly, 300 to 400 µg of mRNA in 100 µL of water was mixed with 100 µL of dimethyl sulfoxide (DMSO) and 200 µL of formamide and layered over a 4-mL 5 to 20% sucrose density gradient in 99% DMSO, 1 mM
In Vitro Translations and Immunoprecipitations

RNA was translated in rabbit reticulocyte lysate (Bethesda Research Laboratories) containing [3H]-leucine, according to the manufacturer's instructions. Anti-VBP antibody was prepared as previously described (Wolpert and Macko, 1991). Indirect immunoprecipitations were performed according to the procedure of Anderson and Blobel (1983). SDS-PAGE was performed with the buffer system of Laemmli (1970). Fluorography was performed with ENHANCE (Du Pont) according to the manufacturer's instructions.

cDNA Synthesis and Cloning into λ gt11

cDNA was prepared from size-fractionated, membrane-bound mRNA with a cDNA synthesis kit (Amersham). cDNA libraries were prepared in λ gt11 with an EcoRI adapter ligation system (Promega). Plaque lifts on nitrocellulose were performed according to standard protocols (St. John, 1990). DNA gel blot analysis, conducted with the 1.2-kb cDNA as a probe, indicated an apparent full-length cDNA for the 100-kD VBP, a second probe, and purified on an HRLC MA7Q anion exchange column (Bio-Rad) with a 30-min, linear gradient of 0.85 M KCl to 1.2 M KCl in 50% formamide and 20 mM potassium phosphate, pH 6.7. Fractions containing the plasmid were pooled and precipitated with isopropanol.

Nucleic Acid Blotting and Labeling and Plasmid Purification

DNA gel blotting onto nitrocellulose membranes was conducted as described by Selden (1991a). RNA gel blotting was performed with glyoxal-DMSO denaturing gels, followed by transfer to nitrocellulose as described by Selden (1991b). DNA was labeled by random oligonucleotide-primed synthesis (Tabor and Struhl, 1991). DNA gel blot analysis, conducted with the 1.2-kb cDNA from the polylinker region of pFTQ19, the insert could be present in either of two orientations relative to the promoter region. The insert with the 5' end (relative to the mRNA) adjacent to the promoter, two recombinant plasmids were selected, each with opposite orientations as determined by restriction digests. A series of bidirectional exonuclease III digestions were performed starting from the polylinker at the 5' end of the insert (relative to the promoter region) in each recombinant plasmid. These digestions were analyzed by DNA gel blotting by probing with the 1.2-kb cDNA. The results were interpreted based on the assumption that the 1.2-kb insert represented the 3' end (relative to the mRNA) because cDNA synthesis had been primed with oligo(dT). This approach identified a plasmid with the correct orientation. The plasmid with the proper orientation was then used as a substrate for limited 5' deletions (relative to the promoter) of ~115, 230, and 345 bp to a total of ~800 bp. When digested into TQ19, transformed into E. coli, and induced by IPTG, these deletion constructs resulted in antibody-positive colonies. The 5'-deleted insert was subsequently cloned into pUC19, which provided a substantial increase in the level of expression of the recombinant protein in E. coli (data not shown).

cDNA Sequence Analysis

The near full-length 3.4-kb cDNA clone was subcloned into a pBluescript SK– vector (Stratagene). Each strand of the clone was sequenced independently using a Sequenase kit (U.S. Biochemicals). T3 and T7 primers complementary to the T3 and T7 promoters of the vector were used in initial reactions to determine the sequence of the 5' ends of the cloned cDNA. Primers (17mers) were then synthesized based on sequence information from the 3' end of the region that had just been sequenced. The primers were then used to prime the next sequencing reaction. The set of clone-specific primers used to determine the entire cDNA sequence were spaced 250 to 300 bp apart along each strand of the cloned DNA. 32P-labeled products of sequencing reactions were electrophoresed on 5% Long Ranger (AT Biochem, Malvern, PA) acrylamide gels in an S2 electrophoresis apparatus (Gibco BRL). Sequence ambiguities were resolved with inosine base analogs. Sequence data were compared to known sequences using IntelGenetics (Mountain View, CA) molecular biology software.

Protein Comparison between Oats and Recombinant E. coli

The 100-kD VBP from oats was extracted and subjected to anion exchange chromatography as described previously (Wolpert and Macko, 1991). Protein prepared in this manner was used for electrophoresis.
The 5' 400-bp exonuclease-deleted fragment, the smallest deletion fragment capable of directing anti-VBP positive expression, was sub-cloned into pUC19 and transformed into E. coli DH5α. The use of pUC19 resulted in much higher expression of the protein than expression in pTQ19. Protein was partially purified from the recombinant cells as follows. Cells were harvested from 100 mL of IPTG-induced overnight culture by pelleting at 1000g. The pellet was resuspended in 40 mL of 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 6 mM β-mercaptoethanol (RB buffer) and centrifuged at 1000g. The pellet was resuspended in 20 mL of RB buffer, frozen at –20°C, thawed, mixed with 100 µL/mL of lysozyme, and incubated at room temperature with gentle shaking for 3 hr. The suspension was homogenized in a glass Dounce homogenizer and centrifuged at 10,400g in a Sorvall HB4 swinging bucket rotor (Du Pont). The pellet was resuspended in RB buffer plus 1% Triton X-100, incubated 1 hr at room temperature with gentle shaking, and centrifuged as before. Finally, the pellet was resuspended in RB buffer plus 1% SDS and used for electrophoresis.

Proteins prepared from oats and recombinant E. coli were subjected to SDS-PAGE on 8% gels. Comparable amounts of the 100-kD VBP protein and recombinant protein were loaded from each source as determined by staining test gels with Coomassie Brilliant Blue R 250. After electrophoresis, the band representing either the oat 100-kD VBP or the recombinant protein was detected by staining for 5 min in 0.1% Coomassie blue in methanol-water-acetic acid (5:5:1). The bands were then cut out of the gel with a razor blade and stored frozen at –20°C until they were used for peptide mapping.

Limited proteolysis was performed according to the methods of Flannery et al. (1989). Briefly, gel slices of oat and recombinant protein were thawed and equilibrated 30 min in 0.125 M Tris-HCl, pH 6.8 (CB buffer) plus 0.1% SDS. Slices were placed in the wells of a 14% polyacrylamide gel, overlaid with 20 µL of CB buffer plus 20% glycerol, and then overlaid with 10 µL of protease in CB buffer plus 10% glycerol. The samples were subjected to electrophoresis until migration had proceeded approximately half way through the stacking gel and the power was turned off. After a 30-min incubation, the power was restored and electrophoresis completed. Protease concentrations used were as follows: 10 µg/mL of Staphylococcus aureus V8 protease (Sigma); 1 µg/mL of subtilisin (Sigma); and 300 µg/mL of chymotrypsin (Sigma).

Gels were then analyzed by protein gel blotting onto nitrocellulose membranes with a semidry blotting apparatus (Bio-Rad) with a buffer of 20% methanol, 25 mM Tris, 192 mM glycine. Proteins were transferred for 1 hr at 3 mA/cm². Blots were developed with anti-VBP antibody as described previously (Wolpert and Macko, 1991).

Species Distribution of the VBP

Chlorella protein extract was prepared as follows. Fifteen milliliters of Chlorella cells (10⁶ cells per mL) were centrifuged at 5000g for 15 min, and the supernatant was discarded. The pellet was resuspended in 3 mL of 62.5 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 5% β-mercaptoethanol, and 10% sucrose and boiled for 5 min. The cell material was then centrifuged at 10,000g, and the supernatant was saved. Protein from all other plants was prepared by homogenizing 0.5 g of leaf tissue per 10 mL of ice-cold 50 mM 3-(N-morpholino)propanesulfonic acid (Mops), 2 mM EDTA, 0.4 M sucrose, 6 mM β-mercaptoethanol, pH 7.5 (homogenization buffer), with a mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 400g for 4 min. The supernatant was then centrifuged at 100,000g for 30 min, the supernatant was discarded, and the pellet was resuspended in homogenization buffer. Pellets from Arabidopsis, tomato, wheat, and maize were resuspended in one-tenth the original volume, and pellets from the other plant extracts were resuspended in one-half the original volume.

E. coli protein extract was prepared by inoculating 50 mL of Luria-Bertani media with 100 µL of an overnight culture of strain DH5α. The cells were harvested after 4 hr by centrifugation at 3000g for 5 min. The cell pellet was washed once with H₂O, pelleted, and resuspended in 0.4 M sorbitol, 25 mM Tris, 10 mM EDTA, pH 8.0 (lysozyme buffer), with 5 mg/mL lysozyme. After 30 min, cells were pelleted as before and washed once with lysozyme buffer before resuspending in 1 mL of ice-cold 100 mM Na₂PO₄, 10 mM EDTA, pH 7.2. Spheroplasts were lysed with a Dounce homogenizer. The cell lysate was then freeze thawed and pelleted in a microcentrifuge, and the supernatant was collected.

Bovine liver was obtained from the Department of Animal Science, Oregon State University. Bovine liver protein extract was prepared by freeze thawing a 0.5-g sample of liver tissue followed by grinding with a mortar and pestle in 1 mL of 50 mM Mops-NaOH, 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.01% BSA, pH 7.2. The sample was then centrifuged for 5 min in a microcentrifuge, and the supernatant was collected.

Gel blot analysis conducted with antibody to the 100-kD VBP was performed on all protein extracts as described previously (Wolpert and Macko, 1991), except that SDS-polyacrylamide gels were soaked in transfer buffer for 2 min prior to transfer and then electroblotted at 1.5 mA per cm² of gel for 90 min onto nitrocellulose using a semidy dye Trans-Blot apparatus (Bio-Rad).

Mitochondria Isolation

Seedlings were homogenized with a chilled mortar and pestle in cold sample buffer (0.3 M mannitol, 10 mM Mops, pH 7.2, 1 mM EDTA, 0.1% defatted BSA) or extraction buffer (0.35 M mannitol, 1 mM EDTA, 0.1% defatted BSA, 0.6% polyvinylpyrrolidone, 30 mM Mops, pH 7.5, 3 mM β-mercaptoethanol). The initial homogenate was pelleted at 10,000g for 10 min, and the pellet was gently resuspended in the same buffer. The resuspended pellet was then overlaid onto Percoll gradients and mitochondria isolated essentially as described by Douce et al. (1987).

ACKNOWLEDGMENTS

We wish to extend our thanks to Blaine Baker for his photographic work. This work was supported in part by Grant No. DCB 90-16871 from the National Science Foundation and a grant from the Center for Gene Research and Biotechnology, Oregon State University. This is Oregon Agricultural Experiment Station Technical Paper 10040.

Received April 20, 1994; accepted June 17, 1994.

REFERENCES


Frey, K.J., Browning, J.A., and Brindeland, R.L.

Kim, Husic, D.W., Husic, H.D., and Tolbert, N.E.


Identification of the 100-kD victorin binding protein from oats.
T J Wolpert, D A Navarre, D L Moore and V Macko
Plant Cell 1994;6;1145-1155
DOI 10.1105/tpc.6.8.1145

This information is current as of October 28, 2017

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