Immunological Evidence for a Cell Surface Receptor of Victorin Using Anti-Victorin Anti-Idiotypic Polyclonal Antibodies

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Anti-victorin anti-idiotypic polyclonal antibodies were raised in rabbits immunized with an anti-victorin polyclonal antibody-ovalbumin conjugate. The presence of anti-victorin anti-idiotypic antibodies in the serum was examined by the inhibition of victorin-horseradish peroxidase (HRP) binding against anti-victorin antibody in a direct enzyme-linked immunosorbent assay (ELISA). Victorin-HRP binding in a direct ELISA was inhibited 84% by addition of a tenfold diluted anti-victorin anti-idiotypic serum. Sera from nonimmunized rabbits did not show significant inhibition. The anti-idiotypic serum diluted 20-fold, induced callose synthesis in protoplasts from victorin-sensitive but not insensitive oat cultivars. When anti-idiotypic antibodies were added simultaneously with 60 pg of victorin per milliliter, they inhibited victorin-induced callose synthesis by 68%. Since anti-idiotypic antibodies are presumed to be too large to enter the cell, these results indicate that victorin acts at the surface of cells.

Cochliobolus victoriae (Meehan & Murphy) Subram & Jain, the fungus that causes victoria blight of oats (*Avena sativa* L.), produces a host-specific toxin, victorin. Both susceptibility to the pathogen and sensitivity to victorin are determined by the dominant allele of the *Vb* gene (Pringle and Scheffer 1964). It has been hypothesized that the *Vb* gene encodes a receptor for victorin and that lack of a receptor is the cause of resistance (Pringle and Scheffer 1964).

Several lines of indirect and direct evidence support the existence of a victorin receptor site or sites; pretreatment with inhibitors of RNA or protein synthesis or reagents that block SH groups reduce the sensitivity of oats to victorin (Gardner and Scheffer 1973; Rancillac *et al.* 1976; Walton and Earle 1985). Pretreatment with heat also induces the loss of sensitivity to victorin (Briggs *et al.* 1984; Bronson and Scheffer 1977). These data indicate that victorin sensitivity requires a constitutive protein, hypothesized as a receptor, with a short turnover time (<12 hr). Wolpert and Macko first reported that an ^{125}I-labeled victorin analog binds covalently to a 100-kDa protein in a genotype-specific manner *in vivo*, but not *in vitro* (Wolpert and Macko 1989). However, we recently found that native victorin binds to 100-, 65-, and 45-kDa proteins, as detected by Western blotting with anti-victorin antibody, in both susceptible and resistant oats, and both *in vivo* and *in vitro* (Akimitsu *et al.* 1992). These binding proteins, which bind victorin covalently, have been considered as possible candidates of victorin receptors (Wolpert and Macko 1989; Akimitsu *et al.* 1992). However, the relationship of the observed victorin binding to the specificity or toxicity of victorin is still unknown.

Anti-idiotypic antibodies raised against the idiotypes of antibodies to biologically important ligands have been used successfully to identify cell surface receptor sites in systems such as the insulin receptor (Sege and Peterson 1978), β-adrenergic receptor (Guillet *et al.* 1985; Schreiber *et al.* 1980), the nicotine receptor (Abood *et al.* 1987), the diphtheria toxin receptor (Rolf *et al.* 1989), and other animal hormones, neurotransmitters, and lymphotrophic viruses and factors (Strosberg 1989). Since the internal image of an anti-idiotypic antibody and the ligand epitope that binds to the receptor will be identical or very similar, the anti-idiotypic antibody also recognizes the ligand binding site of the receptor.

Anti-victorin antibody used in our victorin-binding studies could detect as little as 10 pg of victorin per milliliter in an indirect enzyme-linked immunosorbent assay (ELISA) (Akimitsu *et al.* 1992). In this study, we used this anti-victorin antibody as an immunogen to produce anti-victorin anti-idiotypic antibodies for further examination of victorin binding proteins.

RESULTS

Screening of anti-victorin anti-idiotypic antibodies.

Anti-victorin anti-idiotypic antibodies were assayed by the inhibition of victorin-HRP binding to anti-victorin antibody in a direct ELISA. When victorin was used as a competitor of victorin-HRP binding to anti-victorin antibody in the direct ELISA system, 25 ng of victorin per milliliter inhibited victorin-HRP binding by 50% and 1 μg of victorin per milliliter inhibited victorin-HRP binding by 97.5% (Akimitsu *et al.* 1992). When different concen-

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trations of protein A column-purified serum collected after the third boost were mixed with victorin-HRP, as much as 84% of victorin-HRP binding was inhibited by the addition of 1×10^{-1} diluted serum and at least 4×10^{-3} diluted serum still inhibited 33% of binding (Fig. 1). There was no significant inhibition when preserum was used (Fig. 1). The increase of anti-idiotypic antibody production in serum was correlated with the number of boosts (Fig. 2).

Effects of anti-victorin anti-idiotypic antibody on oat protoplasts.

Victorin induces extracellular callose synthesis in susceptible oat protoplasts (Walton and Earle 1985). About 70% of susceptible oat protoplasts produced callose after 8 hr of incubation with 60 pg of victorin per milliliter, and callose production continued to increase for 24 hr (Figs. 3A and 4). The secretion is host-specific, since no induction occurred in resistant oat protoplasts after the same treatment (Figs. 3B and 4). The 20-times diluted serum (third boost) containing anti-victorin anti-idiotypic antibodies induced callose synthesis in 10% of the susceptible oat protoplasts, but in none of the resistant oat protoplasts (Figs. 3C and 5). The amount of callose produced from susceptible oat cells 18 hr after treatment with anti-idiotypic antibodies was similar to that induced by 6 pg/ml of victorin (Fig. 3C,D). Treatment with a 1:20 dilution of preserum did not induce callose synthesis in either susceptible or resistant oat protoplasts (Fig. 5). When the same concentration of serum containing anti-idiotypic antibodies was added simultaneously with 60 pg/ml of victorin to oat protoplasts, inhibition of callose synthesis was observed (Fig. 4). Callose synthesis was inhibited by 68% after 5 hr (Fig. 4). A 1:20 dilution of preserum had no effect on the response to victorin (Fig. 4). Twenty micrograms of BSA or human Ig per milliliter did not induce callose and also had no effect on the callose secretion induced by 60 pg/ml of victorin (Table 1).

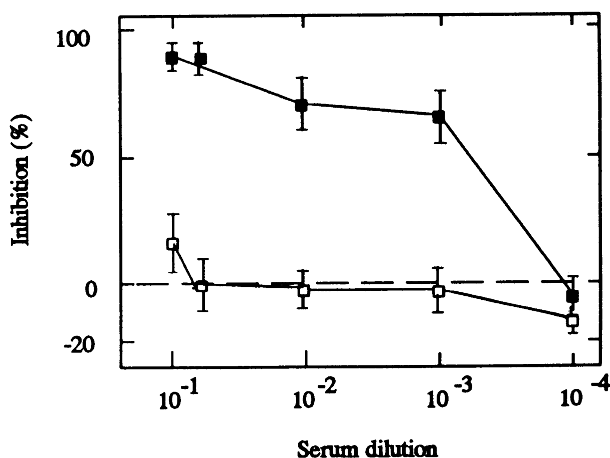

Fig. 1. Inhibition of binding of a victorin-horseradish peroxidase (HRP) conjugate to anti-victorin antibody by anti-victorin anti-idiotypic antibodies in a direct ELISA. IgGs were purified from serum immunized with an anti-victorin antibody-ovalbumin (OVA) conjugate or preserum, and mixed with victorin-HRP. The mixture was added to microtiter wells precoated with anti-victorin antibody. Inhibition of victorin-HRP binding was calculated by the comparison of A_{450} of victorin-HRP plus IgG mixture to victorin-HRP alone. Error bars indicate standard deviation of the mean of six repetitions. ■, antiserum immunized with anti-victorin antibody-OVA; □, preserum.

DISCUSSION

Anti-victorin anti-idiotypic polyclonal antibodies were produced in rabbits immunized with anti-victorin antibody-OVA conjugates. Since the antibody molecule is too large to enter cells, anti-idiotypic antibodies are often used for the determination of cell-surface receptors (Sega and Peterson 1978; Strosberg 1989). Although a large number of anti-idiotypic antibodies have been prepared (Strosberg 1989), this technique has rarely been used in a study of plant-microorganism interactions.

In this study, we examined the effect of anti-victorin anti-idiotypic antibodies on the production of callose from oat cells. Extracellular synthesis of callose from susceptible oat protoplasts is a very sensitive reaction of oat cells to victorin (Walton and Earle 1985). Sixty picograms of victorin per milliliter induced the synthesis of callose by 70% of the cells after 5 hr (Fig. 4) and as little as 6 pg of victorin per milliliter stimulated callose secretion in some cells (Fig. 3D) (Walton and Earle 1985). Although callose synthesis is a sensitive response to victorin, there is no evidence that this is the primary site of action of victorin. Victorin does not have an effect on glucan synthase II (callose synthase) activity in oat homogenates (Akimitsu *et al.* 1992), and low concentrations of victorin also induce other physiological responses, including ethylene (Shain and Wheeler 1975) and avenalumin synthesis (Mayama *et al.* 1986).

The anti-idiotypic antibodies worked weakly as an agonist on susceptible oat protoplasts (Fig. 5), but also worked as an antagonist to the action of victorin (Fig. 4). These results may be explained by the polyclonal nature of these anti-idiotypic antibodies which may consist of several "internal images" of the binding part of the victorin molecule to receptor. Several of the "internal images"

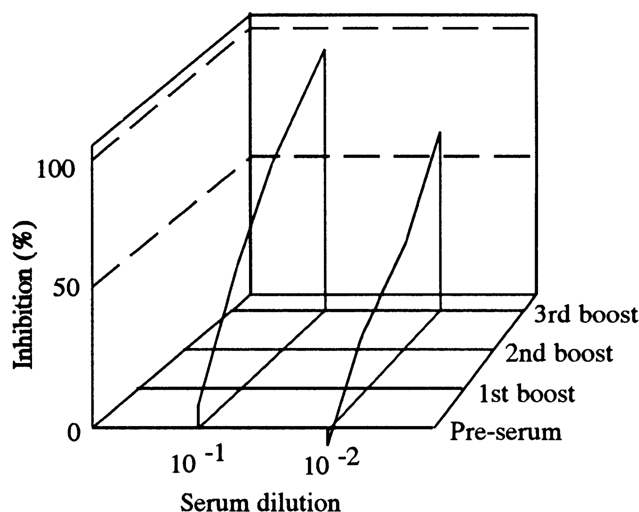

Fig. 2. Stimulation of anti-idiotypic antibody production by immunogen boosts. Production of anti-victorin anti-idiotypic antibodies was examined by inhibition of victorin-HRP binding to anti-victorin antibody in a direct ELISA.

Fig. 3. The production of callose from oat protoplasts induced by victorin or by anti-victorin anti-idiotypic antibodies. Callose was stained with Calcofluor white and observed by fluorescence microscopy after 18 hr of incubation. All magnifications were 400×. **A,** Sensitive protoplasts treated with 60 pg/ml of victorin; **B,** resistant protoplasts treated with 60 pg/ml of victorin; **C,** sensitive protoplasts treated with 20-times diluted serum containing anti-victorin anti-idiotypic antibodies; and **D,** callose synthesis from sensitive protoplasts induced by 6 pg/ml of victorin.

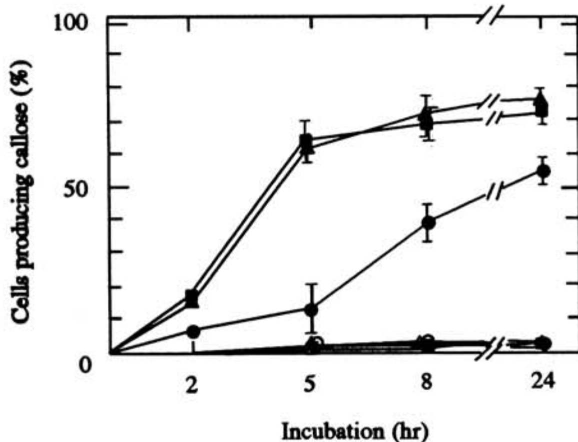

Fig. 4. Inhibition of victorin-induced callose synthesis by anti-victorin anti-idiotypic antibodies. Twenty-times diluted anti-idiotypic serum or preserum was mixed with 60 pg/ml of victorin and treated to 0.5 ml of protoplast solution (4×10^4 protoplasts per milliliter). The number of cells making callose was counted and the percentage of total cells calculated. Error bars indicate the standard deviation of the mean of three repetitions. ●, Victorin, anti-idiotypic serum, susceptible protoplasts; ○, victorin, anti-idiotypic serum, resistant protoplasts; ■, victorin, preserum, susceptible protoplasts; □, victorin, preserum, resistant protoplasts; ▲, victorin, susceptible protoplasts, no serum; △, victorin, resistant protoplasts, no serum.

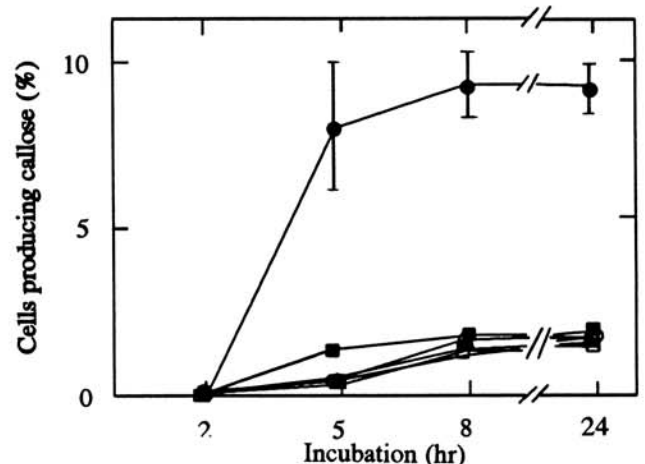

Fig. 5. Induction of callose synthesis from oat protoplasts by anti-victorin anti-idiotypic antibodies. Twenty-times diluted anti-idiotypic serum or the same concentration of preserum was mixed with 0.5 ml of protoplast solution (4×10^4 protoplasts per milliliter). The number of cells making callose was counted and the percentage of total cells calculated. Error bars indicate standard deviation of the mean of three repetitions. ●, Anti-idiotypic antibodies, susceptible protoplasts; ○, anti-idiotypic antibodies, resistant protoplasts; ■, preserum, susceptible protoplasts; □, preserum, resistant protoplasts; ▲, susceptible protoplasts, no serum (control); △, resistant protoplasts, no serum.

carried by the anti-idiotypic antibodies might lead to more than a single type of interaction with the receptor; some anti-idiotypic antibodies could act like victorin to induce callose synthesis, but other anti-idiotypic antibodies could block victorin binding to the receptor. Schreiber *et al.* (1980) reported that polyclonal anti-idiotypic antibodies against β-adrenergic worked as an agonist and also as an antagonist on ligand-receptor interactions (Schreiber *et al.* 1980). They speculated that multiple types of interactions of anti-idiotypic polyclonal antibodies to the β-adrenergic receptor occurred (Schreiber *et al.* 1980), and later found a monoclonal anti-idiotypic antibody which had only agonist properties of the physiological function of the original ligand (Guillet *et al.* 1985).

Our results using anti-victorin anti-idiotypic antibodies suggest that a victorin binding site related to victorin toxicity may exist on the surface of the plasma membrane of susceptible oat cells. The evidence supports the hypothesis that victorin receptor sites are on the plasma membrane of susceptible oats (Briggs *et al.* 1984; Bronson and Scheffer 1977; Gardner and Scheffer 1973; Hanchey and Wheeler 1968; Keck and Hodges 1973; Novacky and Hanchey 1974; Rancillac *et al.* 1976). The data suggest that anti-victorin anti-idiotypic antibody might be a useful tool to examine a cell surface receptor of victorin. However, the production of monoclonal anti-idiotypic antibody will probably be necessary for further examination of the receptor-anti-idiotypic antibody interactions.

MATERIALS AND METHODS

Plant materials.

Park was used as the *C. victoriae*-susceptible and victorin-sensitive oat cultivar, and Korwood was used as the *C. victoriae*-resistant and victorin-insensitive oat cultivar. Oats were grown 20–25 days in a growth chamber at 18° C under fluorescent and incandescent lamps (fluence rate: 140 μmol/m/sec), with a 12-hr photoperiod.

Victorin C preparation.

The major form of victorin, victorin C (Wolpert *et al.* 1988), was isolated from 40 L of culture filtrates of *C. victoriae* (isolate 1146A) by previously described methods (Akimitsu *et al.* 1992; Mayama *et al.* 1986).

Victorin antibody production.

Anti-victorin antibodies were produced in rabbits immunized with victorin-bovine serum albumin (BSA) conjugate as described (Akimitsu *et al.* 1992). For the preparation of anti-victorin antibody-OVA conjugates, anti-victorin polyclonal antibodies were purified using immobilized-protein A and BSA columns (Akimitsu *et al.* 1992).

Table 1. Effect of proteins on victorin-mediated callose synthesis

Treatment[a]	Cells producing callose (%)
Victorin (60 pg/ml)	68.9 ± 5.9
Victorin (60 pg/ml) plus BSA (20 μg/ml)	75.5 ± 6.7
Victorin (60 pg/ml) plus human IgG (20 μg/ml)	69.8 ± 3.5

[a] Concentrations listed are the final concentrations in 0.5 ml of oat protoplast solution (4×10^4 protoplasts per milliliter).

Production of anti-victorin anti-idiotypic antibody:immunogen preparation.

Anti-victorin antibodies purified by immobilized protein A and BSA columns were conjugated with ovalbumin in phosphate-buffered saline (PBS), pH 7.2, with glutaraldehyde (Langone and Bjercke 1989). Briefly, 1 mg of antibodies was dissolved in PBS, pH 7.2, and mixed with 1 mg of ovalbumin. Eighty microliters of glutaraldehyde (25% commercial solution from Sigma) was added to the solution and incubated for 45 min at room temperature. The reaction was stopped by the addition of 200 μl of 2 M lysine, and the solution was dialyzed against four changes of PBS overnight at 4° C.

Immunization.

The antibody-OVA conjugate (0.5 mg) was dissolved in 1 ml of PBS and mixed with 1 ml of Freund's complete adjuvant. The mixture was injected intradermally into three female New Zealand white rabbits, and boosted three times intramuscularly at 28-day intervals with 0.5 mg of conjugate in 1 ml of PBS plus 1 ml of Freund's incomplete adjuvant. Blood was taken from rabbits 12 days after each injection, and serum was isolated (Harlow and Lane 1988). The serum was purified by protein A column chromatography (Pierce/ImmunoPure IgG Purification kit), and the presence of anti-victorin anti-idiotypic antibodies was determined.

Screening for anti-victorin anti-idiotypic antibody.

The anti-victorin anti-idiotypic antibody was assayed by the inhibition of victorin-HRP binding to anti-victorin antibodies in a direct ELISA (Akimitsu *et al.* 1992; Strusberg 1989). Conjugation of victorin to HRP was described previously (Akimitsu *et al.* 1992). Twenty micrograms of anti-victorin antibody purified by both immobilized protein A and BSA columns in 100 μl of PBS (200 μg/ml) was placed in wells of a microtiter plate and incubated overnight at 4° C. The antibody solution was removed, and the well was washed with PBS-Tween 15 times. The plate was then incubated with 300 μl of PBS-Tween for 0.5 hr at 37° C to block unbound binding sites and washed with PBS-Tween five times. Victorin-HRP (diluted $1{:}10^4$ from the stock solution [Akimitsu *et al.* 1992]) or victorin-HRP plus different concentrations of sample serum (purified by protein A column) or victorin-HRP plus different concentrations of victorin, in 1 ml of PBS-Tween, were placed on each well and incubated for 1 hr at 37° C. The plate was then washed 15 times with PBS-Tween, and 100 μl of HRP substrate (0.4 mM tetramethylbenzidine, 0.004 % H_2O_2, in 50 mM sodium acetate, pH 6.0) was added. After 30 min of incubation at room temperature, the reaction was stopped by addition of 2 M H_2SO_4 and absorbance at 450 nm was read with a Bio-Tec ELISA reader.

Protoplast preparation.

Sterile protoplasts were prepared by the methods described by Schaeffer and Walton (1990). Green oat leaves (500 mg) were sterilized with 10% bleach, abraded with Carborundum, and incubated at 26° C for 3 hr in 2% (w/v) Cellulysin in SCM buffer (0.5 M sorbitol, 10 mM $CaCl_2$, and 40 mM Mes, pH 5.5) with gentle shaking. Released protoplasts were filtered through 80-μm nylon screen mesh, washed twice with SCM (pH 5.8), and collected by centrifugation at $100 \times g$ for 5 min. The protoplasts were washed with SCM (pH 5.8) by centrifugation two more times, and resuspended in SCM (pH 5.8) at a final concentration of 4×10^4 protoplasts per milliliter.

Anti-victorin anti-idiotypic antibody treatment of oat protoplasts.

Protoplasts (0.5 ml of 4×10^4 protoplasts per milliliter) were placed in wells of 24-well Falcon multiwell plates (Becton-Dickinson), and mixed with victorin (60 pg/ml) and/or 1:20 diluted serum containing anti-idiotypic antibodies. BSA, human IgG (protein standard from Sigma), and preserum, were used as controls. The protoplasts were incubated in the dark for various periods at room temperature, and callose production was observed by staining with a 1:1 mixture of 0.1% Calcofluor white and SCM (pH 5.8) and viewing under a Zeiss epifluorescence microscope (filter set G365) at a final magnification of 400×.

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