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THE INDUCTION AND DEGRADATION OF CALLOSE

By

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ABSTRACT

THE INDUCTION AND DEGRADATION OF CALLOSE

By

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Victorin, the host-selective toxin produced by *Cochliobolus victoriae*, induces mesophyll protoplasts of susceptible varieties of *Avena sativa* to produce an extracellular polysaccharide (EPS). EPS was determined to be (1-3) β-D-glucan (callose). EPS was also produced by these cells in response to $AlCl_3$, $GdCl_3$, $InCl_3$, YCl_3, or $YbCl_3$, but not $ScCl_3$, $GaOCl_3$, $FeCl_3$,$CrCl_3$, or $LaCl_3$. Digitonin but not other detergents induced EPS synthesis. Calcium channel agonists, antagonists, chelators, ionophores, or calmodulin inhibitors tested neither prevented callose induction caused by victorin nor induced callose synthesis themselves. Removal of calcium from the external medium neither induced callose production nor inhibited victorin-induced callose biosynthesis. Protoplasts of victorin resistant *Avena sativa*, and also *Triticum aestivum* and *Hordeum vulgare* produce an EPS, presumably callose, in response to greater than 10 μM $AlCl_3$. However, EPS production in response to Al ions did not appear to be related to Al ion tolerance at the level of whole plants.

If stress-induced callose biosynthesis protects against pathogen invasion, then the ability to degrade callose might be important to a successful pathogen. To test this, the gene, EXG1, encoding an exo β (1-3) glucanase was isolated from the maize pathogen *Cochliobolus carbonum* and sequenced. Southern

blotting suggested a single copy of the gene. The gene was disrupted by transformation-mediated homologous recombination. The mutant lacked the protein and associated enzyme activity. Culture filtrates of the mutant still contained 44% of wild type activity when grown on oat bran cereal. This residual β (1-3) glucanase enzyme activity coeluted from hydrophobic interaction chromatography HPLC with β (1-3)(1-4) (mixed-linkage) glucanase activity and therefore is due to different enzymes. The mutant grew at wild type rates on sucrose and oat bran cereal but grew poorly on β (1-3) glucan compared to wild type. The mutant was equally pathogenic on susceptible maize cultivars.

The larger the island of knowledge,

The wider the horizon of wonder.

Unknown

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ABBREVIATIONS

BAPTA	bis-(o-aminophenoxy)-ethane-N,N,N'N', tetraacetic acid
CIAP	calf intestine alkaline phosphatase
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EPS	extracellular polysaccharide
Hepps	N-hydroxyethylpiperazine-N'-propanesulfonic acid
HIC	hydrophobic interaction chromatography
Mes	2-(N-morpholino) ethanesulfonic acid
n.d.	not determined
RI	refractive index
SCM	0.5 M sorbitol, 10 mM $CaCl_2$, 40 mM Mes
SE	standard error

TFA	trifluoroacetic acid
t-Glc	non-reducing terminal Glc
TMB-8	8-(N,N'-diethylamino)octyl-3,4,5-trimethoxybenzoate

CHAPTER 1

INTRODUCTION

INTRODUCTION

The plant cell wall is a major barrier to adverse environmental conditions. During an interaction between a plant and a potential pathogen, the pathogen can use a number of mechanisms to invade plant tissue, colonize, and obtain nutrients from plant tissues. Pathogens can enter through natural openings of the plant such as stomates (i.e. *Uromyces appendiculatus*: Hoch *et al.* 1987), or through the cell wall by mechanical force (Howard & Ferrari 1989) or by enzymatic degradation (Collmer & Keen 1986). Pathogens can produce cell-wall degrading enzymes, host-selective toxins, or nonspecific toxins which can affect plants. In response to pathogen attack, plants can i) alter the composition of the cell walls, ii) produce enzymes such as chitinases and glucanases that can degrade the pathogen's cell wall (Mauch *et al.* 1988), iii) produce phytoalexins (Darvill & Albersheim 1984) or iv) produce a hypersensitive response (Klement 1982).

Callose as a Component of Papillae

One commonly observed plant response to wounding or attempted pathogen penetration is the production of specialized structures called wound plugs (in response to wounding) (Currier 1957) or papillae (in response to

pathogen attack) (Aist 1976). Papillae are wall-like depositions between the cell wall and the plasma membrane (Aist 1976). Callose (β (1-3) glucan) is one of the most common, although perhaps not universal components, of papillae (Aist 1976; Cadena-Gomez & Nicholson 1987; Hinch & Clarke 1982). Papillae also can contain pectin, suberin, lignin, proteins and silicon (Aist 1976). The trigger for papillae induction is not known. Signals can result from mechanical damage (Bushnell 1972) or chemical signals produced by the pathogen (Aist & Israel 1977a,b; Skipp *et al.* 1974).

Based on several lines of evidence, it has been proposed that papillae may prevent pathogen invasion. For example, Bushnell and Bergquist (1975) observed papillae formation by barley in response to attempted infection by the wheat pathogen, *Erysiphe graminis* f. sp. *tritici* and therefore concluded that papillae were important resistance mechanisms of barley to the wheat pathogen. Vance and Sherwood (1976) determined that reed canarygrass as well as many other plants in the Gramineae (Sherwood & Vance 1980) produce papillae in response to attempted infection by noninfecting pathogens such as *Stemphylium botryosum*, *Helminthosporium maydis* race T, and an incompatible isolate of *Curvularia lunata*. When papillae formation was inhibited by cycloheximide, these normally noninfecting pathogens could then successfully penetrate and produce haustoria. The conclusion from this study was that papillae are an important component of non-host resistance.

Because callose is a frequent component of papillae, callose is also thought to protect against pathogen invasion. For example, the nonhost,

maize, produces callose-containing papillae in response to *Phytophthora cinnamomi* and invasion is not significant, whereas in the host, *Lupinus angustifolius*, papillae are not produced and infection is successful (Hinch & Clarke 1982). Stumm and Gessler (1986) observed that cucumber leaves exhibiting induced resistance produced callose-containing papillae (Binder *et al.* 1990) earlier than plants lacking induced resistance and that these papillae were more efficient in preventing penetration by *Colletotrichum laginarium*. Significantly more callose is produced by *Glycine max* resistant to *Phytophthora megasperma* f. sp. *glycinea* during attempted infection than by the susceptible variety and this callose is deposited adjacent to the hyphae of the pathogen (Bonhoff *et al.* 1987). In addition, treatment of resistant barley with the callose synthesis inhibitor 2-deoxy-glucose resulted in increased infection by *Erysiphe graminis* (Bayles *et al.* 1990). However, in the presence of this callose synthesis inhibitor, the frequency of haustorium formation by *Uromyces vignae* on the nonhost, *Phaseolus vulgaris*, did not increase, although no callose was produced (Perumalla & Heath 1989). Therefore, evidence suggests that papillae are important in some disease interactions.

If callose-containing papillae can act as barriers to pathogens, degradation of papillae might be important for a pathogen to successfully infect its host. Van Hoof *et al.* (1991) recently purified an exo β (1-3) glucanase from the maize pathogen *Cochliobolus carbonum*. If callose and papillae are important in plant protection and if a successful pathogen must enzymatically degrade them, then perhaps the exo β (1-3) glucanase described by van Hoof

et al. (1991) is an essential pathogenicity factor. To test this hypothesis, the gene encoding this exo β (1-3) glucanase has been isolated and a strain of this fungus has been constructed that is specifically mutated in this exo β (1-3) glucanase (Chapter 2).

Induction of Callose Biosynthesis

Callose is found as a minor component of plant cell walls, observed in pollen tubes (Rae *et al.* 1985), sieve plates (Currier 1957), and phloem plugs (Mangin 1889). However, callose is rapidly produced *in vivo* at sites of damage from mechanical wounding (Eschrich & Currier 1964; Brett 1978; Currier 1957), pathogen invasion (Aist 1976), heavy metals (Fincher & Stone 1981), and heat stress (Majunder & Leopold 1967; McNairn 1972).

In an effort to better understand the regulation of callose biosynthesis, the enzyme that synthesizes callose has been studied. Originally callose synthase activity was detected in membrane fractions during efforts to identify cellulose-synthesizing enzymes (Delmer 1983; Mullins 1990). Cellulose-synthesizing enzymes are relatively inactive and therefore have been difficult to analyze. High activity of callose synthase can be obtained from many plant tissues even if callose is not observed in the tissue from which the membranes have been isolated. Therefore, cells have tremendous latent ability to make callose. Callose synthase must be tightly controlled and can be triggered by wounding, including biochemical extraction techniques.

It has been hypothesized that the enzymes responsible for callose and

cellulose biosynthesis are the same enzyme (Delmer 1977; Jacob & Northcote 1985). Callose and cellulose synthesis are rarely observed at the same time or in the same location within the cell. In the cell plate, for example, callose is deposited before cellulose (Fulcher *et al.* 1975), and in pollen tubes, callose is present in the primary wall and cellulose in the secondary wall (Rae *et al.* 1985). Callose is rarely observed in intact cells but cellulose biosynthesis has never been observed *in vitro* (Amor *et al.* 1991).

UDP-Glc is the substrate for callose synthase (Tsai & Hassid 1973; Feingold *et al.* 1958; Brett 1978). However, several compounds stimulate enzyme activity. For example, Ca^{2+} ions and β-glucosides such as cellobiose decrease the K_m for UDP-Glc and increase the V_{max} of the reaction (Delmer 1987b). However, the β-glucoside is not incorporated into the final product (Hayashi *et al.* 1987). Digitonin also increases enzyme activity and although it contains a β-glucoside linkage, digitonin cannot substitute for cellobiose in the assay (Hayashi *et al.* 1987). In addition, the β-glucoside glucosyl dioleoyl diglyceride, isolated from mung bean, stimulates callose synthase activity 20-fold (Callaghan *et al.* 1988). Although callose synthase activity can be enhanced by these various compounds *in vitro*, it is not clear if they have any regulatory role *in vivo*.

To obtain more information about enzymes involved in callose biosynthesis, several laboratories are trying to purify the enzymes. This has proven to be quite difficult because the enzymes are membrane-bound and are composed of several subunits. More progress has been made by studying β-

glucan synthesis in bacteria and fungi. The genes encoding β (1-4) glucan-synthesizing enzymes of the bacterium *Acetobacter xylinum* have been identified as an operon of four genes which have been cloned and sequenced (Wong *et al.* 1991; Saxena *et al.* 1990,1991). Cyclic diguanylic acid has been identified as an activator *in vivo* of *A. xylinum* β (1-4) glucan synthase (Ross *et al.* 1990) and polyclonal antibodies have been produced to the catalytic subunit (Wong *et al.* 1990). These polyclonal antibodies cross-react to plant membrane proteins (Mayer *et al.* 1991). The peptides of cotton that cross-react to these antibodies also bind cyclic diguanylic acid and contain amino acid similarity to the *A. xylinum* protein (Amor *et al.* 1991).

Girard and Fevrè have developed the Oomycetous fungus *Saprolegnia monoica*, as a model system for studying both β (1-3) glucan synthase and β (1-4) glucan synthase. The β (1-4) glucan synthase is stimulated by phosphorylation based on several lines of evidence, i) enzyme stimulation by ATP but not non-phosphorylating analogues, ii) labelling of peptides by [^{32}P]ATP, and iii) reduction of β (1-4) glucan synthase activity by phosphatase treatment (Girard & Fevrè 1991b). Either the synthesizing enzyme or an activator of the enzyme may be phosphorylated. In addition, the β (1-4) glucan synthase of *S. monoica* is activated by cyclic diguanylic acid, an activator of *A. xylinum* β (1-4) glucan synthase; therefore, the enzymes of these two organisms may be related (Girard *et al.* 1991). Girard and Fevrè (1991a) have also identified a stimulator of β (1-3) glucan synthase from membranes of *S. monoica* which has a molecular weight greater than 20 kD and is associated

with a protein that is not necessary for enzyme stimulation. This may be a glycoside activator of *S. monoica* but it is not related to the GTP-dependent protein required for *Saccharomyces cerevisiae* β (1-3) glucan synthase (Kang & Cabib 1986).

Efforts to purify plant β (1-3) glucan synthases have resulted in the enrichment of several peptides (Wasserman *et al.* 1992). Subunits have been partially purified by solubilization of the enzyme from the membranes with detergents (Wasserman *et al.* 1991), product entrapment to enrich for peptides bound to the glucan (Hayashi *et al.* 1987; Frost *et al.* 1990), photoaffinity labelling of enzyme subunits with 5-azido-UDP-Glc (Frost *et al.* 1990), and identifying peptides bound to callose synthase inhibitors (i.e. formaldehyde, 5' diphospho-pyridoxal (Mason *et al.* 1990) and chlorpromazine (B.P. Wasserman; personal communication)). Sufficient purification of some subunits has been obtained to try to clone the genes encoding these subunits (B.P. Wasserman; personal communication) allowing analysis of the callose synthesizing genes and their regulation.

Ca^{2+} ions improve callose synthase activity and in some cases are required for activity. Kauss (1987) proposed that callose synthase may be regulated by Ca^{2+} influx, perhaps by Ca^{2+} channels. Since the activation by Ca^{2+} is reversible, the effects appear to be due to the allosteric effects of Ca^{2+} on the enzyme rather than covalent modification (Kauss 1987).

Walton and Earle (1985) determined that victorin-sensitive oat mesophyll protoplasts produce an extracellular polysaccharide (EPS) in the presence of low

concentrations of victorin. In Chapter 3 this EPS is identified as callose. Because victorin causes symptoms of calcium deficiency (Marinos 1962: Hanchey *et al.* 1968), added calcium alleviates the symptoms caused by victorin and *C. victoriae* (Doupnik 1968), and because Ca^{2+} levels may regulate the callose-synthesizing enzyme (Kauss 1987), calcium channel agonists, calcium channel antagonists, calcium chelators, and a calmodulin inhibitor [also an inhibitor of callose synthase (B.P. Wasserman; personal communication) and Mg^{2+}-dependent phosphatidate phosphohydrolase activity (Walton & Possmayer 1989)] were tested for effects on EPS biosynthesis (Chapter 3).

Callose production in response to victorin is related to victorin sensitivity and *C. victoriae* susceptibility. Since $AlCl_3$ also induces callose in these protoplasts, the relationship between EPS production and Al tolerance of oats, barley, and wheat was tested (Chapter 4).

Callose synthase activity appears to be latent *in vivo* in uninduced tissues. Perhaps the enzyme is constitutively present but its activity is regulated by the plant. If this is the case, then the amount of callose synthase activity isolated from membranes of induced tissue will not differ from that of uninduced tissue. This was examined using protoplasts to study the callose synthase activity in microsomes of victorin-treated and untreated protoplasts (Appendix 1).

CHAPTER 2

The exo β (1-3) glucanase produced by *Cochliobolus carbonum* is required for wild type growth on β (1-3) glucan but not for pathogenicity of maize.

ABSTRACT

The sequences of the N-terminus and of an internal peptide of the exo β (1-3) glucanase of *Cochliobolus carbonum* were used to design degenerate oligonucleotides. Genomic DNA and these oligonucleotides were used in a polymerase chain reaction (PCR) to amplify a 1.1 kb fragment. This fragment was used as a probe to isolate the genomic copy of the gene, *EXG1*. The gene was sequenced. The gene was disrupted by transformation-mediated homologous recombination using a plasmid containing a fragment from the coding region and hygromycin resistance as a dominant selectable marker. Disruption of the exo β (1-3) glucanase gene was shown by genomic Southern blotting. When grown on oat bran cereal, the culture filtrate from this mutant still contained 44% of the wild type β (1-3) glucanase activity. This enzyme activity was due to enzymes other than the EXG1 gene product. These other β(1-3) glucanases coeluted from hydrophobic interaction chromatography-HPLC with β (1-3)(1-4) (mixed-linkage) glucanase activities. The mutant caused disease symptoms similar to wild type isolate 2R15 on susceptible maize. The mutant was unable to grow in culture as well as the wild type with β (1-3) glucan as the sole carbon source.

INTRODUCTION

Plant cell walls act as a defense or barrier to invasion by pathogens. A number of cell wall-degrading enzymes have been identified from plant pathogens including cellulases, xylanases, arabinanases, mannanases, and galactosidases (Bateman 1964; Bateman & Bassham 1976; Cooper 1984; Anderson 1978; Van Etten & Bateman 1969). These enzymes have been studied to understand their role in aspects of pathogenesis, not only in initially penetrating plant tissue but also in obtaining nutrients, colonizing plant tissues, causing disease symptoms, and inducing defense responses within the host plant. Pectolytic enzymes play a role in plant tissue maceration in soft rot diseases caused by *Erwinia* species (Collmer & Keen 1986). Pectolytic enzymes can also release elicitors from the cell wall and thereby induce phytoalexin biosynthesis (Lee & West 1981; Davis *et al.* 1984) and other plant defenses (Bishop *et al.* 1984; Collmer & Keen 1986). Wood-decaying fungi produce cell wall degrading enzymes which, in some cases, allow the pathogen access to nutrients from dead tissue (Liese 1970).

In several examples, molecular genetic approaches have been used to test the importance of particular enzymes in pathogenicity. Cutinase had been previously determined to be necessary for penetration of plant tissue by

Fusarium solani f.sp. *pisi* based on use of cutinase-specific inhibitors (Maiti & Kolattukudy 1979). However, disruption of this cutinase gene did not affect the pathogenicity or virulence of this pathogen on pea (Stahl & Schafer 1992). Scott-Craig *et al.* (1990) tested the involvement of endo-polygalacturonase in pathogenesis by targeted gene disruption. Frequently more than one pathogen-produced enzyme degrades a particular substrate making it difficult to assess the importance of a particular enzyme activity without multiple gene disruptions.

Plant cell wall polysaccharides can be part of an active defense response of plants. *De novo* formation of papillae in response to attempted infection has been correlated with resistance in some diseases (Bushnell & Bergquist 1975; Hinch & Clarke 1982; see Chapter 1). Callose is a major component of many papillae (Aist 1976). If callose biosynthesis is an important defensive barrier against pathogen invasion (Smith 1900; Aist 1976) then it might be beneficial to a pathogen to be able to degrade callose.

An exo β(1-3) glucanase capable of degrading callose has been purified from *Cochliobolus carbonum* (van Hoof *et al.* 1991). The *EXG1* gene, encoding the exo β (1-3) glucanase, has been cloned and disrupted in *C. carbonum* to determine the role of this enzyme in the infection of maize by this pathogen.

MATERIALS AND METHODS

Fungal cultures. *Cochliobolus carbonum* isolate 2R15 (recently renamed isolate SB111) was stored and grown in still culture as described (Walton & Cervone 1990). Growth of mycelia for protoplast and DNA isolation was by the method of Scott-Craig *et al.* (1990). Fungal growth for isolation of β (1-3) glucanase was as previously described (van Hoof *et al.* 1991) on MS medium containing 0.2% sucrose and 1% oat bran cereal (Quaker oat bran cereal; Quaker Oats Co., Chicago, IL: referred to as oat bran in the text). MS medium contained, per liter, 2 g yeast extract, 0.181 g $MgSO_4$, 0.149 g KCl, 1 g $(NH_4)_2SO_4$, 0.65 g KH_2PO_4, 1 ml trace element stock (per 100 ml 0.1 g $MnSO_4 \cdot H_2O$, 0.1 g boric acid, 0.01 g $CuSO_4$, 0.01 g $ZnSO_4 \cdot H_2O$, and 2 g $FeSO_4$). Growth of *C. carbonum* isolate 2R15 and the exo β (1-3) glucanase mutant were compared in 30 ml MS medium in 250-ml flasks supplemented with 2% sucrose, 1% oat bran, or 1% laminarin (Sigma).

Isolation of the Exo β(1-3) Glucanase Gene. Peptide sequences of the N terminus (AIVDGYWLNDLSGK) and an internal fragment (SKPQYETL) of the exo β(1-3) glucanase (van Hoof *et al.* 1991) were reverse translated and degenerate oligonucleotides were synthesized (Biochemistry Macromolecular Synthesis Lab, MSU). Oligonucleotide 1 was a 128-fold degenerate 17-mer, TAYTGGYTNAAYGAYYT synthesized as the coding strand; and oligonucleotide 2 was a 64-fold degenerate 17-mer, GTYTCRTAYTGNGGYTT, synthesized as

the noncoding strand of the internal peptide fragment. (N indicates A,C,G, or T; Y indicates C or T; R indicates A or G;) These oligonucleotides (100 pmoles) were used as primers in the polymerase chain reaction (PCR; Perkin-Elmer Cetus DNA Thermal Cycler;Perkin-Elmer Corp., Norwalk, CT) using 100 ng *C. carbonum* isolate 2R15 genomic DNA as the template. The reactions also contained 50 μM each of dATP, dCTP, dGTP, and dTTP, 10 μl 10x buffer, and 1 U *Taq* polymerase. PCR conditions were as follows: 94°C for 3 min to denature the template, 40 cycles of denaturation at 94°C for 1 min., annealing of primers at 50°C for 2 min, and extension at 72°C for 3 min. This was followed by 72°C for 7 min. to complete DNA extension. DNA was size fractionated by agarose gel electrophoresis in 1x TAE buffer (Sambrook *et al.* 1989). The 1.1 kb PCR product was used as a probe to screen a genomic library. Hybridizing plaques were isolated and inserts from these phage particles were cloned by standard methods (Sambrook *et al.* 1989).

DNA Methods. Fungal DNA was isolated by the method of Yoder (1988). DNA was transferred from agarose to Zetaprobe (Bio-Rad; Richmond CA) by the method of Reed and Mann (1985). DNA probes were labelled by the primer extension method of Sambrook *et al.* (1989). Southern blots were prehybridized and hybridized at 65°C using 10^5-10^6 cpm/ml of probe (Sambrook *et al.* 1989). The *C. carbonum* isolate 2R15 genomic library was made by standard procedures using lambda EMBL 3 (Scott-Craig *et al.* 1990) and was screened by the method of Sambrook *et al.* (1989).

DNA Sequencing. DNA sequence was determined by the dideoxy-method (Sanger *et al.* 1977) using Sequenase (United States Biochemicals) and the manufacturer's protocol using double stranded DNA prepared by the method of Sambrook *et al.* (1989). DNASIS software was used for sequence analysis.

Transformation-Mediated Gene Disruption. The vector used for transformation (pHG1) was made by cloning a fragment of pUCH1 containing the gene encoding hygromycin phosphotransferase (Turgeon *et al.* 1987) into Bluescript (Stratagene) containing an internal 0.3 kb *Cla* I fragment of the exo β (1-3) glucanase gene. Protoplasts were isolated from mycelial cultures of *C. carbonum* isolate 2R15 methods similar to Panaccione *et al.* (1988) using 1% Novozym 234 and 1% Driselase. These protoplasts were transformed with pHG1 as previously described (Scott-Craig *et al.* 1990) after complete digestion at the unique *Bgl* II site internal to the *Cla* I fragment.

Analysis of Putative Transformants. Single conidia of transformants were selected on 10 μg/ml hygromycin to ensure nuclear homogeneity. Maize cultivar Pr x K61 plants were inoculated with conidial suspensions (10^4 conidia/ml) of *C. carbonum* isolate 2R15 and two independent transformants (T1 and T3). Disease progression was evaluated daily. The fungus was reisolated from the plant to ascertain that the transformants had remained resistant to hygromycin after growth on the plants.

Purification of the Exo β (1-3) Glucanase Enzyme. Exo β(1-3) glucanase was purified from *C. carbonum* 2R15 wild type and transformant by methods of van Hoof *et al.* (1991). The concentrated fraction was separated by HIC-HPLC using the method of van Hoof *et al.* (1991) with the modification that the concentrated fraction was not fractionated by anion exchange HPLC prior to HIC (hydrophobic interaction: Biogel TSK-Phenyl-5PW, BioRad, Richmond, CA)-HPLC.

β (1-3) Glucanase assays. Enzyme activity was determined by the method of van Hoof *et al.* (1991) using the reducing sugar assay of Lever (1972). Routinely, the assays were started by adding 60 μl 1% laminarin to 300 μl containing 50 μl of enzyme preparation and all other components of the reaction. After 20 min incubation, 25 μl of the reaction was added to 1.5 ml p-hydroxy benzoic acid hydrazide. Mixed-linkage glucanase was determined by the same method using β(1-3)(1-4)glucan (Sigma) as the substrate.

Chemicals. *Taq* DNA polymerase, and restriction enzymes were obtained from Boehringer Mannheim. Novozym 234 was purchased from Novo Nordisk Biochemicals. Driselase, DEAE cellulose, and *p*-hydroxy benzoic acid hydrazide were purchased from Sigma. Hygromycin was obtained from Calbiochem, and ampicillin from US Biochemicals. [^{32}P]-alpha-dCTP, 800 Ci/mmol, and [^{35}S]-alpha-dATP, 1400 Ci/mmol, were purchased from New England Nuclear. All other supplies were molecular biology or reagent grade.

RESULTS

Probe and gene isolation. Degenerate oligonucleotides 1, TAYTGGYTNAAYGAYYT, and 2, GTYTCRTAYTGNGGYTT, amplified a single 1.1 kb PCR product using *C. carbonum* 2R15 DNA as the template at a 50°C primer annealing temperature. This PCR product hybridized to a single band in each lane of a genomic blot of *C. carbonum* isolate 2R15 DNA (Fig. 1). The single band of hybridization in each lane indicated that a single 1.1 kb PCR product was obtained lacking these restriction sites and is consistent with a single copy of this sequence in this fungal strain. This PCR product was then used to isolate genomic clones and a 9.8 kb *Bam* HI and 2.8 kb *Sal* I fragment that hybridized to the PCR product were subcloned. Both strands of a 2.8 kb region were sequenced (Fig. 2). The oligonucleotides used for PCR and the DNA sequences encoding the peptide sequences were located within this fragment 1.18 kb apart (Fig. 2). The amino acid sequence was deduced from the nucleic acid sequence in the reading frame containing the peptide fragments determined directly (boxed amino acids, Fig. 2). The peptides used to design the PCR primers are in different reading frames and there are two stop codons between them. Therefore, an intron may be spliced from within the PCR-amplified region. Based on the consensus sequence of introns (Ballance 1991) there are potential splice sites which would remove a 152 bp intron and place

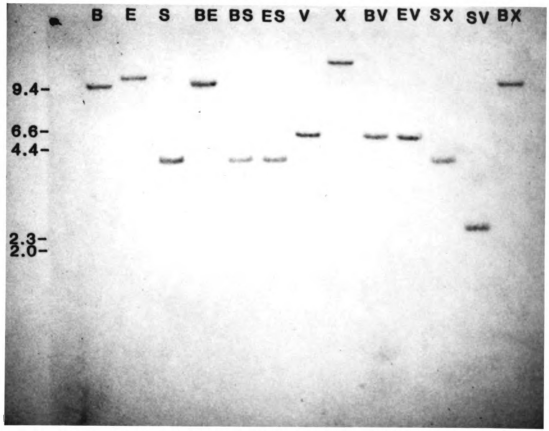

Figure 1: Genomic Southern blot of *Cochliobolus carbonum*. Isolated DNA was digested with various restriction enzymes, separated by gel electrophoresis, blotted to nylon membrane and probed with the 1.1 kb PCR product. B = *Bam* HI, V = *Eco* RV, X = *Xho* I, E = *Eco* RI, S = *Sal* I.

Figure 2: Nucleotide sequence of the genomic copy of the exo β (1-3) glucanase gene. Restriction sites: Bold = intron splice consensus sequences, boxes = peptide sequences determined directly, underline = putative introns. Nucleotide sequence is written above the amino acids it encodes.

```
   1 AAAAGACGCAAGACATGGAGCCACCCAAATGGCCGCATGTGTAGTCGCGCACCAAGAGTG  60
  61 CAAACCATACCTCCGTGTGACCTTTGAGCGGGAGCCAGTAAGGAATCCTGACCGGGTCCT 120
 121 GCCTGTTTGTGAGCTGCTCCGGGGAGTGTTCATCCTCGATCCACCCGGTGTAGTGAACGT 180
 181 CGCGTTTACACGATGATAAAACCGGGGCTACAAGGAATGTGTTGACAGAATACTTAAATA 240
 241 GAGAGCATCACCTGCCTTTGAGACATTCCCATCTCTACTACGCTCTACATTTCCTTGTAT 300
 301 AAAGTGTCCTTCAATTCACTTTGCTGCTCAATCGGAAATCTGCTGGTCTGAAGTTCACGA 360
 361 GCATGCGTTTTTCTTCTTTGCTCGCCTGCCTAGGTGCAGTCGGCATTCAAGCCGCTGCTA 420
       M  R  F  S  S  L  L  A  C  L  G  A  V  G  I  Q  A  A  A  I
 421 TACGTACGAAGATGTCTTCCGCTTTACAACTCCTGGTTACTGACACTTTGTAGCATTCCA 480
       R  T  K  I
 481 AAGGCGTGTTGATAACACTACCGACAGTGGAAGTCTTGATGCTGCTCAAGCTGCGGCTGC 540
                                 G  S  L  D  A  A  Q  A  A  A  A
 541 TATAGTCGATGGCTACTGGCTAAACGATCTCTCCGGCAAAGGCAGAGCCCCTTTTAACAG 600
       I  V  D  G  Y  W  L  N  D  L  S  G  K  G  R  A  P  F  N  S
 601 CAACCCGAACTACAAGGTCTTCCGAAATGTCAAGGATTACGGAGCGAAGGGTAAGCAATT 660
       N  P  N  Y  K  V  F  R  N  V  K  D  Y  G  A  K  A
 661 TTTTTTCACATTGATCTTGAGGATATAACTAACCGATTTGTAGGTGACGGTGTCACTGAC 720
 721 GACTCTGATGCCTTCAACCGTGCCATCTCTGACGGCAGCCGTTGCGGCCCATGGGTTTGC 780
 781 GACTCGTCAACTGACAGCCCAGCTGTTGTTTACGTGCCTTCTGGAACCTATCTCATCAAC 840
                               V  V  Y  V  P  S  G  T  Y  L  I  N
 841 AAGCCCATCATCTTCTACTACATGACTGCTCTCATCGGCAACCCCCGCGAACTTCCCGTC 900
       K  P  I  I  F  Y  Y  M  T  A  L  I  G  N  P  R  E  L  P  V
 901 CTCAAGGCTGCATCTTCACTCCAAGCTCTTGCTCTGATCGACGGAAGCCCCTACAGCAAC 960
       L  K  A  A  S  S  L  Q  A  L  A  L  I  D  G  S  P  Y  S  N
 961 CAAAACGGTGAGCCCGGCTGGATCTCAACCAACTTGTTCTTGCGCCAAATCCGCAACTTG 1020
       Q  N  G  E  P  G  W  I  S  T  N  L  F  L  R  Q  I  R  N  L
1021 ATCATCGATGGCACTGCTGTTGCACCAACATCGGGTTTCCAGGCTATCCATTGGCCCGCC 1080
       I  I  D  G  T  A  V  A  P  T  S  G  F  Q  A  I  H  W  P  A
1081 TCTCAAGCCACCACGATCCAAAATGTCAAGATCCGCATGACACAGGCGTCCAACTCTGTT 1140
       S  Q  A  T  T  I  Q  N  V  K  I  R  M  T  Q  A  S  N  S  V
1141 CACGCTGGTATCTTTGTCGAGAATGGATCTGGCGGTCATATGGCCGACCTCGACATCACC 1200
       H  A  G  I  F  V  E  N  G  S  G  G  H  M  A  D  L  D  I  T
1201 GGTGGTCTGTACGGCATGAACATTGGCAATCAGCAGTTCACCATGCGTAACGTCAAGATC 1260
       G  G  L  Y  G  M  N  I  G  N  Q  Q  F  T  M  R  N  V  K  I
1261 TCCAAGGCTGTCGTCGGTATCTCACAAATCTGGAACTGGGGCTGGCTGTATTCTGGTCTC 1320
       S  K  A  V  V  G  I  S  Q  I  W  N  W  G  W  L  Y  S  G  L
1321 CAGATCAGCGACTGCGGCACTGCTTTCTCCATGGTTAACGGTGGCTCTGCTGGCAAACAG 1380
       Q  I  S  D  C  G  T  A  F  S  M  V  N  G  G  S  A  G  K  Q
1381 GAGGTTGGCTCCGCCGTCATCATCGATTCTGAGATTACCAACTGCCAAAAGTTTGTCGAC 1440
       E  V  G  S  A  V  I  I  D  S  E  I  T  N  C  Q  K  F  V  D
1441 TCAGCATGGTCGCAGACCAGCAACCCTACCGGTTCCGGCCAGCTCGTCATTGAGAACATC 1500
       S  A  W  S  Q  T  S  N  P  T  G  S  G  Q  L  V  I  E  N  I
1501 AAGCTCACCAACGTTCCCGCTGCTGTTGTCAGCAATGGCGCCACTGTCCTCGCTGGCGGC 1560
       K  L  T  N  V  P  A  A  V  V  S  N  G  A  T  V  L  A  G  G
1561 TCTCTTACCATCCAGACCTGGGGTCAGGGCAACAAGTACGCACCCAACGCATCTGGCCCA 1620
       S  L  T  I  Q  T  W  G  Q  G  N  K  Y  A  P  N  A  S  G  P
1621 TCCAAGTTCCAGGGCGCCATCAGCGGTGCCACTCGTCCCACTGGTCTCCTCCAGAACGGC 1680
       S  K  F  Q  G  A  I  S  G  A  T  R  P  T  G  L  L  Q  N  G
1681 AAGTTCTACTCCAAGTCGAAGCCACAGTACGAGACTCTCAGCACTTCAAGCTTTATCAGT 1740
       K  F  Y  S  K  S  K  P  Q  Y  E  T  L  S  T  S  S  F  I  S
1741 GCCCGCGGTGCAGGTGCAACCGGTGATGGTGTCACTGACGACACACGCGCCGTCCAGGCT 1800
       A  R  G  A  G  A  T  G  D  G  V  T  D  D  T  R  A  V  Q  A
1800 GCCGTCACTCAGGCCGCGTCTCAGAACAAGGTCCTCTTCTTCGAGCACGGCGTCTACAAG 1860
       A  V  T  Q  A  A  S  Q  N  K  V  L  F  F  E  H  G  V  Y  K
1861 GTCACCAACACCATCTACGTTCCCCCCGGCTCCCGCATGGTCGGTGAGATCTTCTCCGCC 1920
       V  T  N  T  I  Y  V  P  P  G  S  R  M  V  G  E  I  F  S  A
1921 ATCATGGGCTCTGGCAGCACCTTCGGCGACCAAGCAACCCCGTCCCCATTATCCAAATCG 1980
       I  M  G  S  G  S  T  F  G  D  Q  A  T  P  S  P  L  S  K  S
1981 GCAAGCCCGGCGAGTCCGGCAGCATCGAGTGGTCCGACATGATTGTCCAGACCCAAGGCG 2040
       A  S  P  A  S  P  A  A  S  S  G  P  T  *
```

Figure 2

these peptides in the same reading frame without stop codons between them (Fig. 2). The exact ATG start of the protein cannot be determined because the mature protein appears to be post-translationally processed; there are stop codons in the reading frame near the N-terminus of the mature protein. However, there are potential intron splice sites which would remove 74 bp, bringing a Met into frame with the other peptides and removing stop codons (Fig. 2).

Gene disruption. *C. carbonum* isolate 2R15 was transformed using the vector pHG1. Hygromycin-resistant transformants were analyzed for changes in the restriction pattern of DNA isolated from the transformants and wild type (Fig. 3A). The results are consistent with multiple insertions at the *Bgl* II site (Fig. 3B,C) and disruption of the gene.

Properites of transgenic fungus. a: enzyme activity in the mutant. Culture filtrates from the transformant still contained the ability to degrade laminarin (β (1-3) glucan). After concentration and dialysis of the culture filtrate, the transformant contained 44% of the activity of the wild type per unit protein. The culture filtrate was chromatographed on DEAE-cellulose with a 0.4 M KCl step gradient. All fractions (including the void volume of the wild type) containing enzyme activity were collected, pooled and 0.5 mg protein was fractionated by HIC-HPLC. A peak of UV absorption and a peak of enzyme activity eluted at 33 min in the wild type enzyme preparation (Fig. 4A), consistent with the properties of the enzyme when purified by van Hoof *et al.* (1991). This peak of UV absorption was absent in the transformant and the

Figure 3: A: Genomic Southern blot of transformants. DNA was isolated from *C. carbonum* wild type (WT) and transformant (T1; T3), digested with *Eco* RV or *Hin*d III, blotted to nitrocellulose and probed with the 0.3 kb *Cla* I fragment of the exo β (1-3) glucanase gene. B: Restriction pattern expected in wild type and transformant after *Eco* RV digest or C: *Hin*d III. ▬ = 0.3 kb *Cla* I fragment used as the probe.

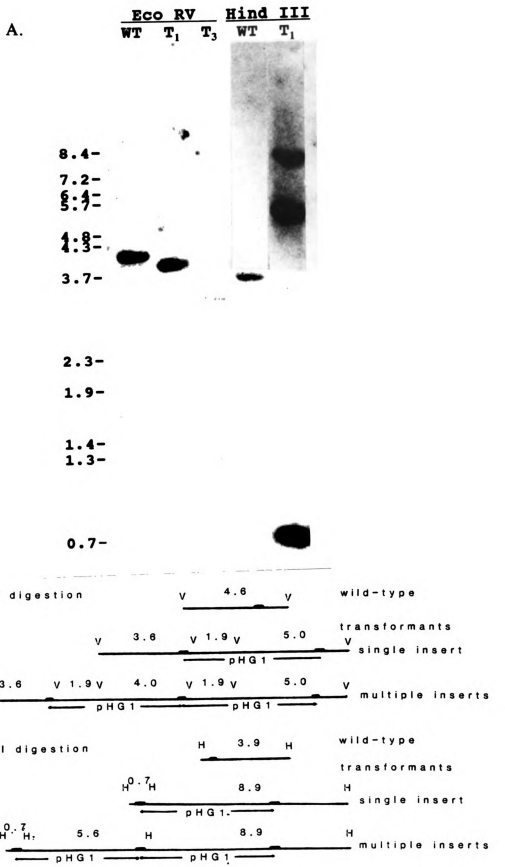
A.
Eco RV
Hind III
WT
T1
T3
WT
T1
8.4-
7.2-
6.4-
5.7-
4.8-
4.3-
3.7-
2.3-
1.9-
1.4-
1.3-
0.7-
B. Eco RV digestion
V
4.6
V
wild-type
transformants
V
3.6
V
1.9
V
5.0
V
single insert
pHG1
V
3.6
V
1.9
V
4.0
V
1.9
V
5.0
V
multiple inserts
pHG1
pHG1
C. Hind III digestion
H
3.9
H
wild-type
transformants
H
0.7
H
8.9
H
single insert
pHG1
H
0.7
H
5.6
H
8.9
H
multiple inserts
pHG1
pHG1

Figure 3

Figure 4: Characterization of β (1-3) glucanase activities by HIC-HPLC. Wild type (A) and mutant T1 (B) enzyme preparations were fractionated by HIC-HPLC and fractions were assayed for β (1-3) glucanase activity. C) Mixed-linkage glucanase activity of HIC-HPLC fractions. (-●- = wild type; x = mutant; enzyme activity in $OD_{410}/\mu l$ fraction).

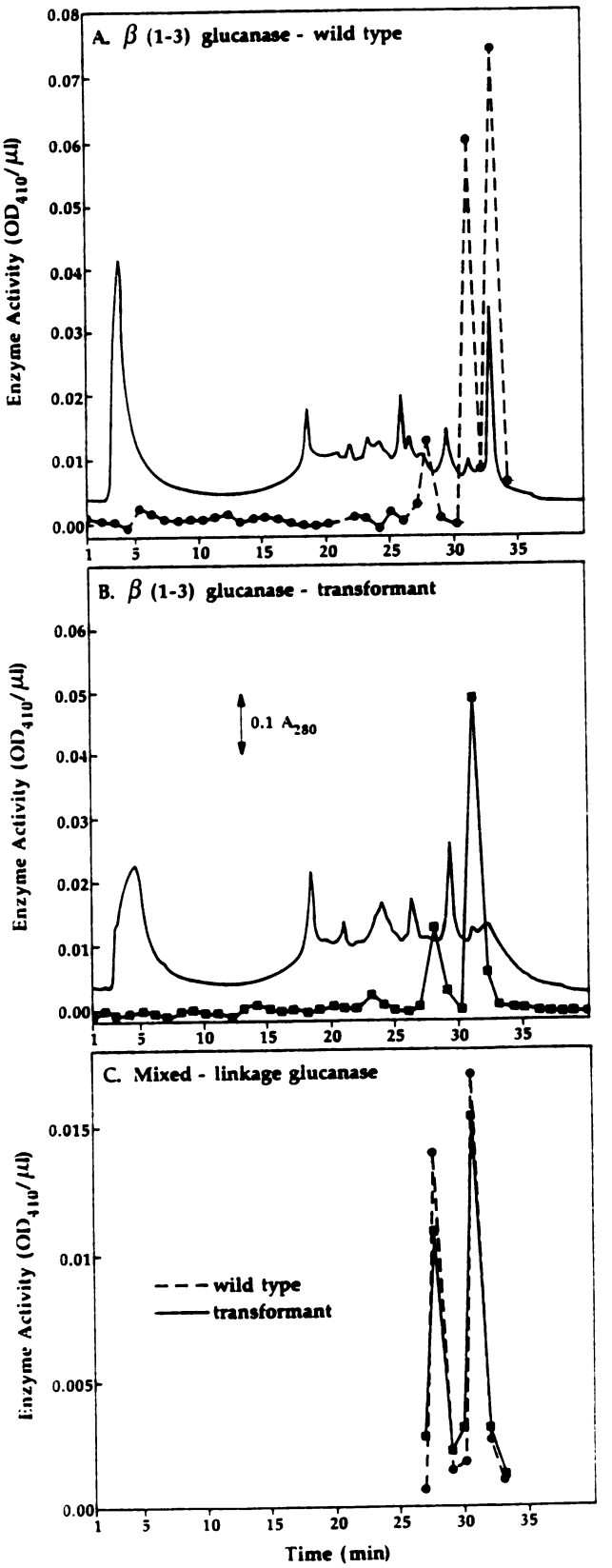
A. β (1-3) glucanase - wild type
B. β (1-3) glucanase - transformant
C. Mixed - linkage glucanase
Enzyme Activity (OD410/μl)
0.1 A280
wild type
transformant
Time (min)

Figure 4

33 min fraction of the transformant contained only 2% of the enzyme activity of the wild type (Fig. 4B). Therefore, the gene has been successfully disrupted and the transformant does not produce the exo β (1-3) glucanase that was purified. However, in assaying the other fractions from HIC-HPLC, activity was located in fractions eluted at 28 min and at 31 min in both wild type and mutant preparations (Fig. 4A,B). These fractions also contain β (1-3)(1-4) (mixed-linkage) glucanase activity which is absent in the 33 min fraction (Fig. 4C). Therefore, the β (1-3) glucanase activity observed in the mutant is not from the exo β (1-3) glucanase activity but from different enzymes that coelute with β (1-3)(1-4) glucanases.

b: disease. Susceptible maize plants that had been inoculated with mutants (T1, T3) or wild type (2R15) showed no differences in the rate of development or severity of disease (Fig. 5). Since mycelia isolated from the plants infected with the mutants were still hygromycin resistant, this disease progression was not due to reversion of the mutant.

c: growth in culture. The mutant grows equally well as wild type on 1% oat bran or 2% sucrose (Fig. 6). The mutant's ability to grow on 1% laminarin was reduced by 65% (Fig. 6,7).

DISCUSSION

The gene encoding the exo β (1-3) glucanase produced by *C. carbonum* has been disrupted by transformation-mediated homologous recombination.

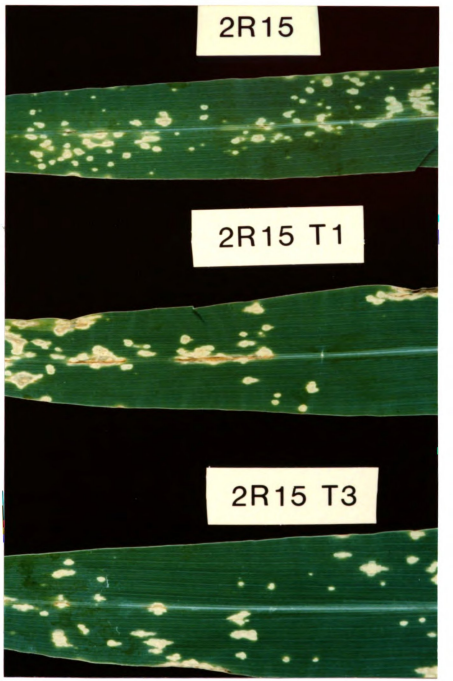

Figure 5: Pathogenicity of wild type and mutants. Two-week old Pr x K61 plants were infected with conidia of *C. carbonum* isolate 2R15 (wild type) or mutants (2R15 T1 or 2R15 T3). The photograph shows the plant 6 d following infection.

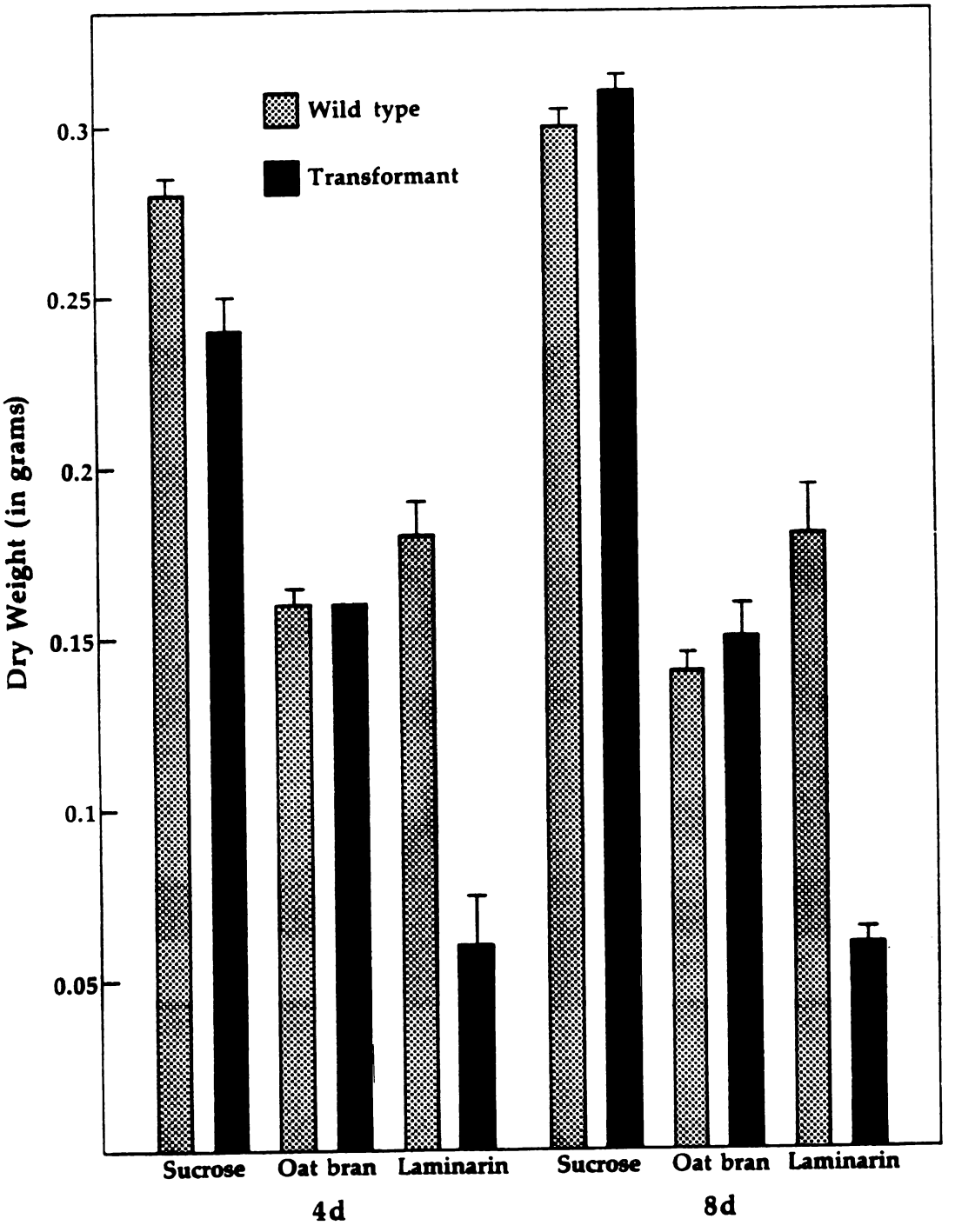

Figure 6: Comparison of growth of mutant and wild type on several media. MS media containing as carbon source 2% sucrose, 1% oat bran, or 1% oat bran were inoculated with wild type (2R15) or mutant (T1) and growth as dry mat weight was compared after 4 d and 8 d ($\pm$1 SE (n=2)).

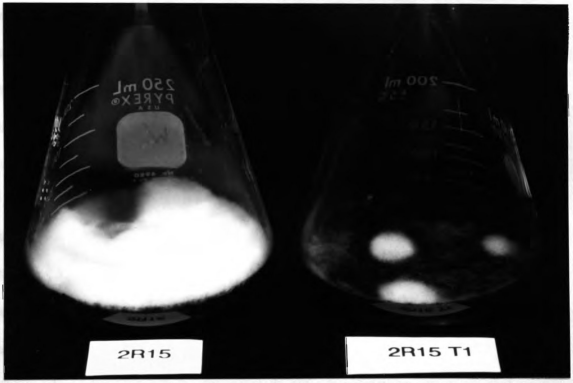

Figure 7: Growth of mutant and wild type *C. carbonum* on laminarin. MS media containing 1% laminarin as carbon source was inoculated with *C. carbonum* isolate 2R15 or mutant T1. The photograph shows fungal growth after 5 d.

This enzyme is not required for pathogenicity of *C. carbonum* on susceptible varieties of maize (Fig. 5) but is necessary for wild type growth on β (1-3) glucan (Fig. 6,7). However, other enzymes are produced by this fungus which are capable of degrading laminarin in enzyme assays and coelute with mixed linkage (β (1-3)(1-4)) glucanases. These mixed-linkage glucanases may be capable of degrading both β (1-3) glucans and mixed-linkage glucans or there may be two different enzymes in these fractions. The remaining β (1-3) glucanase activity in the mutant may be important to the plant-pathogen interaction and therefore compensate for the absence of the exo β (1-3) glucanase during infection of susceptible maize.

The β (1-3) glucanases (possibly the mixed-linkage glucanases) clearly cannot convert laminarin to compounds capable of supporting fungal growth (Fig. 6,7). However, the mutant grows as well as the wild type on oat bran as a carbon source (Fig. 6). If the mixed-linkage glucanase can degrade β (1-3) glucans then one reason for the decreased growth may be that the mixed-linkage glucanases are induced when the fungus is grown on oat bran, which contains mixed-linkage glucans, but cannot be induced by β (1-3) glucans alone. Alternatively, the mixed-linked glucanases may not be able to degrade the laminarin to saccharides small enough to be metabolized.

The 28 min and 31 min fraction containing β (1-3) glucanase and β (1-3)(1-4) glucanase activity were not identified during the original purification of the exo β (1-3) glucanase (van Hoof *et al.* 1991). However, the overall yield of β (1-3) glucanase was only 32% (van Hoof *et al.* 1991). Some of the lost

enzyme activity may have been due to these other enzymes. Since "enzyme preparations up until the anion exchange HPLC fractionation did degrade mixed-linked glucan" (van Hoof *et al.* 1991), these enzymes must have been separated from the enzyme preparation fractionated prior to HIC-HPLC. Analysis of the mutant allowed the identification of these other enzymes able to degrade β (1-3) glucans (Fig. 4C). In addition, we were not trying to purify the enzyme and therefore reduced the losses in enzyme activity while trying to identify the remaining laminarin-degrading enzymes.

Exo β (1-3) glucanase is not required for pathogenicity of *C. carbonum* on maize. However, we cannot conclude that the ability to degrade β (1-3) glucans is not important to pathogenicity. As a result of the disruption of the exo β (1-3) glucanase it is now known that other enzymes can degrade this substrate. Since many monocots cell walls contain mixed-linkage glucan (Stinard & Nevins 1980; Henry & Stone 1982), these enzymes may also play a role in cell wall degradation if they are capable of degrading both substrates. The ability to degrade both a component of the cell wall and a potential defense response polysaccharide may make these enzymes important to pathogenicity.

CHAPTER 3

Victorin induces mesophyll protoplasts of susceptible oat varieties to produce an extracellular (1-3) β-D-glucan (callose)

ABSTRACT

Victorin induces mesophyll protoplasts of susceptible varieties of oats to produce an extracellular polysaccharide (EPS) (Walton & Earle 1985. Planta 165:407). A number of other chemicals were tested for ability to induce EPS synthesis. The trivalent salts of Al, Gd, In, Y, and Yb also induced EPS synthesis but those of Sc, Fe, Ga, Cr, and La do not. Digitonin induced EPS synthesis but four other detergents tested do not. Based on chemical analysis, EPS induced by $AlCl_3$ is composed solely of glucose in 1-3 linkages. This is consistent with previous data that Sirofluor and Calcofluor fluoresce in the presence of EPS and that EPS is degraded by laminarinase (β (1-3) glucanase) and crude hemicellulase but not pectinase (polygalacturonase). EPS is a (1-3) β-D-glucan (callose). A number of studies have suggested that Ca^{2+} is involved in the response of susceptible oats to *Cochliobolus victoriae* and to victorin. Furthermore, callose synthase has been hypothesized to be regulated by Ca^{2+}. Calmodulin inhibitors, calcium channel agonists and antagonists, calcium chelators, and calcium ionophores neither prevented the induction caused by victorin nor induced EPS synthesis by themselves. Removal of calcium from the external medium did not affect EPS production. Overall, no evidence for the direct involvement of Ca^{2+} in victorin-induced callose synthesis was found.

INTRODUCTION

Cochliobolus (Helminthosporium) victoriae produces a cyclic peptide toxin, victorin (Wolpert *et al.* 1985a,b; Gloer *et al.* 1985), which has the same specificity as the fungus on *Avena sativa* containing the dominant allele at the *Vb* locus (Meehan & Murphy 1946; 1947; Luke & Wheeler 1955). Infection by *C. victoriae* results in the disease Victoria blight. This genotype of oat is not only susceptible to *C. victoriae* but is resistant to certain races of *Puccinia coronata* (Litzenberger 1949; Welsh *et al.* 1953). The genetic linkage of these two traits is complete (Murphy & Meehan 1946).

One of the major symptoms of Victoria blight is wilting. Increases in membrane permeability can result in wilting. Wheeler and Black (1962; 1963) showed that infected or victorin-treated susceptible tissue lost electrolytes more rapidly than resistant or uninfected susceptible plants and that these losses were detectable within 5 min. Since permeability increased so rapidly, Luke *et al.* (1966) proposed that the plasma membrane was the primary site of victorin action. By ultrastructural studies Luke *et al.* (1966) observed a dark-staining material between the plasma membrane and the cell wall of victorin-treated susceptible root and leaf tissue. The deposition of this material was followed by separation of the plasma membrane from the cell wall and

disruption of the internal membrane systems. This led to the conclusion that the primary mode of action is the inner surface of the cell wall or the outer surface of the plasma membrane. Later, Hanchey *et al.* (1968) showed that the plasma membrane retains its unit structure even after severe victorin-induced damage. However, research by Samaddar and Scheffer (1971) indicates that the plasma membrane is disrupted by victorin. The electron density of the cell wall following permanganate fixation is increased by victorin treatment and this occurs prior to the deposition of material between the cell wall and the plasma membrane (Hanchey *et al.* 1968).

Rancillac *et al.* (1976) observed that mesophyll protoplasts of Victoria-derived oat varieties (*C. victoriae*-susceptible) were sensitive to victorin. In addition, root cap cells and root protoplasts of victorin-sensitive plants were equally sensitive to victorin (Hawes 1983). Concentrations of 0.1 ng victorin/ml caused sensitive cells to burst, while mesophyll protoplasts of resistant varieties withstood much higher concentrations with no deleterious effects (Walton & Earle 1985). In addition, susceptible protoplasts produced an extracellular polysaccharide (EPS) in response to low concentrations of victorin (Walton & Earle 1985). This EPS fluoresced in the presence of Calcofluor and was degraded by hemicellulase but not pectinase. Walton and Earle (1985) concluded that this EPS produced by protoplastsis is likely the same material that was observed by Hanchey (1980) which was produced by the intact plant in the presence of victorin. This EPS is deposited between the cell wall and the plasma membrane in the intact plant but is dispersed into the

medium when produced by protoplasts.

Protoplasts offer several advantages as a system for studying the effects of victorin on EPS production. For example, the absence of a cell wall avoids any complications associated with its cation exchange properties and facilitates quantitation and analysis. In addition, protoplasts can be easily monitored for cell death in response to different treatments. Control and experimental treatments are performed on a homogenous population of cells and test compounds have immediate and unhindered access. Therefore, the complex disease interaction between two organisms, *C. victoriae* and *A. sativa*, can be simplified to a low molecular weight toxin and individual mesophyll cells, respectively.

Symptoms of Victoria blight (which include wilting, chlorosis, and necrosis) resemble those of calcium deficiency (Goodman 1972; Hanchey *et al.* 1968; Marinos 1962; Saftner *et al.* 1976). In addition, Ca^{2+} ions can suppress symptoms of victorin toxicity (Doupnik 1968) and resistant tissue contains higher Ca^{2+} levels than infected susceptible tissue (Luke & Barnett 1974). One of the major effects of victorin is to stimulate electrolyte leakage due to increased membrane permeability (Wheeler & Black 1963). In addition, calcium is important in the maintenance of membrane integrity (Marinos 1962; Jones & Lunt 1967). Based on these connections the role of calcium ions in Victoria blight has received considerable study.

To obtain a better understanding of the mode of action of victorin, the structure of EPS was determined. A number of chemicals were tested for

ability to induce EPS and the role of Ca^{2+} ions in EPS production was investigated. Portions of this research have been previously published (Schaeffer & Walton 1990).

MATERIALS AND METHODS

Plant Varieties and Growth Conditions. Seeds of oat (*Avena sativa* L.) cv Park (sensitive to victorin), were planted in soil composed of equal parts of peat moss, vermiculite, and perlite and grown in a growth chamber under fluorescent and incandescent lamps (fluence rate of photosynthetically active radiation: 140 μmol/m^2·sec) for a 12 h photoperiod at 18°C for 20 to 30 days.

Protoplast preparation. Sterile protoplasts were prepared by methods modified from Walton and Earle (1985). Leaves (500 mg) were abraded with carborundum and incubated at 26°C for 3 h in 2% (w/v) Cellulysin in SCM (0.5 M sorbitol, 10 mM $CaCl_2$, and 40 mM Mes), at pH 5.5. Released protoplasts were filtered through 80-μm nylon screen mesh, washed twice with SCM, pH 6.0, and collected by centrifugation at 100 *g*. The protoplasts were resuspended in SCM, pH 6.0, at a final concentration of 40,000 protoplasts/ml, and dispensed at 0.5 ml/well into 24-well Falcon multiwell plates (Becton-Dickinson).

Microscopy. After 24 h incubation, protoplasts were visually observed for EPS

production by staining with a 1:1 mixture of 0.1% Calcofluor white in SCM, pH 6.0, or 0.2% Sirofluor in 2% K_3PO_4 and viewing under a Zeiss epifluorescence microscope (filter set G 365) at a final magnification of 400x. Photographs were taken with Kodak Plus-X film. Protoplast survival was evaluated by exclusion of Evan's blue from the cytoplasm.

[^{14}C]Glucose Incorporation Immediately after dispensing protoplasts into the multiwell plates, 0.25 μCi sterile D-[U-^{14}C]Glc in SCM, pH 6.0, was added to each well. The plates were sealed with parafilm and incubated at room temperature (22°C) in darkness for 24 h. The protoplasts were then transferred to test tubes. The wells were washed twice with either 1.0 ml water (in experiments to determine total incorporation of [^{14}C]Glc) or 1.0 ml 80 mM citric acid, pH 3.0, (if proteins were to be removed subsequently with an ion exchange resin), and the washes were transferred to the same tube. The decrease in osmotic pressure caused the protoplasts to burst allowing the cellular debris to be separated from the EPS.

To remove proteins, 1.0 ml of suspended Dowex 50W cation exchange resin equilibrated with 80 mM citric acid pH, 3.0, was added to each tube containing the protoplasts and two washes. The tubes were rocked for 2 h and, after allowing the resin to settle, the supernatants were transferred to 20-ml tubes with one 1.0 ml (80 mM citric acid, pH 3.0) wash of the resin. After addition of ethanol to a final concentration of 70% (v/v, assuming additive volumes), the samples were stored overnight at 4°C, and then collected by

filtering through Whatman GF/A glass fiber filters using a Millipore model 1225 sampling manifold. The filters were rinsed twice with cold 70% ethanol and the retained radioactivity was measured using a scintillation counter. The Dowex treatment decreased the background incorporation of untreated protoplasts but large differences in EPS production were observed with or without the removal of proteins.

Chemical analysis of EPS. For composition and linkage analysis, twelve wells of protoplasts of each treatment were pooled and the protein removed with cation exchange resin as above. The contents of three wells were filtered after precipitation in 70% ethanol to determine the amount of [^{14}C]Glc incorporated per well. The remaining nine samples were filtered through one Whatman GF/A filter and, for composition analysis, the retained EPS was hydrolyzed with 3 ml of 2 N TFA at 124°C and 15 p.s.i. for 80 min. The TFA and water were removed by partial evaporation under N_2 followed by lyophilization. The sample was dissolved in 500 μl H_2O, filtered through a 0.2 μm filter, lyophilized again, and dissolved in 250 μl H_2O.

Sugars were separated on an Aminex HPX-87P HPLC carbohydrate analysis column with a mixed resin de-ashing pre-column (Biorad, Richmond CA) at 85°C, and detected by refractive index (RI) and an in-line radioactivity flow detector (Radiomatic Flo-one Beta). The mobile phase was H_2O flowing at 0.6 ml/min. Unknowns were identified by comparison of retention time to retention times of standards determined by RI (glucose, arabinose, mannose,

xylose, cellobiose, cellotriose) and by radioactivity (glucose, arabinose). Retention times were corrected for the volume of the tubing between the RI and radioactivity detectors.

Linkage analysis of EPS was performed by methylation of EPS following the method of Blakeney and Stone (1985). The methylated EPS was then acid hydrolyzed, reduced with $NaBH_4$, and acetylated with acetic anhydride. The alditol acetates of the partially methylated sugars were separated by GC using a 15 m DB-225 megabore column (J and W Scientific, Folsom, CA) programmed from 150 to 220°C at a rate of 5°C/min. The radioactivity detector received 10 parts and the flame ionization detector received 1 part. Unlabelled methylated cellobiose and laminaribiose were added to the EPS sample as standards in order to identify the peaks from the radioactivity detector.

Chemicals. Cellobiose, cellotriose, EGTA, sorbitol, $AlCl_3$, nifedipine, verapamil, and Dowex 50W cation exchange resin (100-200 mesh, 2% crosslinkage) were obtained from Sigma. $InCl_3$ was purchased from Fluka, YCl_3 and $ScCl_3$ from Pfaltz & Bauer, TFA from Pierce, (±)-Bay K 8644 (catalog number B-112) from Research Biochemicals, and $YbCl_3$, $GdCl_3$, and $GaOCl_3$ from Alfa Research Chemicals. The isomer calcium channel agonist and antagonist +/-202-791 were obtained from Sandoz. D-[U-^{14}C]Ara, 4.0 mCi/mmol, and D-[U-^{14}C]Glc, 258.5 mCi/mmol, were from ICN and NEN, respectively.Calcofluor white from American Cyanamid used in previous studies (Walton & Earle 1985) was used

for fluorescent microscopy. "Fluorescent Brightener 28" (F 6259) from Sigma resulted in poor fluorescence of EPS. Victorin was purified by the method of Walton and Earle (1984). Sirofluor was a gift from Deborah Delmer, Hebrew University, Jerusalem, Israel. Chlorpromazine was a gift from Bruce Wasserman, Rutgers University, NJ.

RESULTS

Oat mesophyll protoplasts synthesized an extracellular polysaccharide (EPS) in the presence of 60 pg victorin/ml at pH 6.0. This EPS fluoresced under UV radiation when stained with Calcofluor white, which is specific for β-glucans (Fig. 1), or Sirofluor, a derivative of aniline-blue specific for (1-3) β-D-glucans (Evans & Stone 1984). Because preliminary tests showed that $AlCl_3$ induced visible amounts of EPS, further studies were undertaken to determine the concentration of $AlCl_3$ necessary for EPS production and protoplast toxicity. Twenty-four hour treatment with 200 μM $AlCl_3$ induced maximal EPS production when observed by microscopy or measured by incorporation of [^{14}C]Glc into ethanol-insoluble product (Fig. 2; Schaeffer & Walton 1990; Chapter 4). At this concentration of $AlCl_3$ 62% of the protoplasts were still viable after 24 h. The increase in EPS production was 10 to 20-fold over that of the controls after removal of labelled proteins by cation exchange (Schaeffer & Walton 1990). At 1 mM $AlCl_3$, less EPS was produced after 24 h and fewer than 2% of the protoplasts survived after 24 h treatment.

Other trivalent cations and metal salts were tested to determine if the

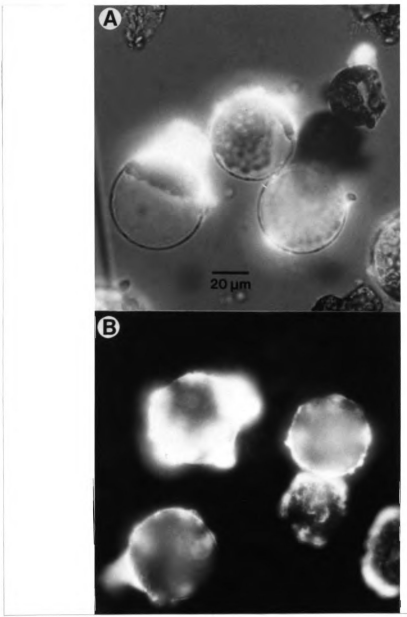

Figure 1. EPS production by oat cv Park mesophyll protoplasts treated with 60 pg victorin /ml for 24 h. A: Protoplasts were photographed under a combination of visible light and UV irradiation after staining with Calcofluor white. B: Protoplasts were stained with Calcofluor and photographed under UV alone.

trivalent charge alone is sufficient to induce EPS. Some, but not all trivalent cations tested, induced EPS (Fig. 2). All were tested at several concentrations but the active compounds typically induced maximal EPS production at 200 μM [$Al_2NH_4(SO_4)_2$ was maximally active at 100 μM]. Of the trivalent ions tested, gadolinium, indium, yttrium, and ytterbium were active, but none were as active as aluminum, as measured both by staining with Calcofluor white and by [^{14}C]Glc incorporation (Fig. 2). $AlCl_3$, $ScCl_3$, $GaOCl_3$, and $Al_2NH_4(SO_4)_2$ were toxic to protoplasts at concentrations of 1 mM. In addition, 200 μM $CuSO_4$ induced visible EPS and was lethal at 1 mM.

The monosaccharide composition of EPS induced by victorin and $AlCl_3$ was analyzed by ion exchange HPLC after complete acid hydrolysis. Glucose was the only sugar detected in EPS (Fig. 3). In this experiment, 90% of the radioactivity injected onto the HPLC was recovered in the eluant. Therefore, EPS does not contain significant quantities of charged sugars such as uronic acids.

The linkage of glucose in the polymer was determined by methylation analysis of radiolabelled EPS (Fig. 4). The radioactive permethylated alditol acetates of EPS were separated by GC and the retention times of the radioactive components were compared with the retention times of permethylated alditol acetates of the unlabelled standards, laminaribiose (3-Glc) and cellobiose (4-Glc). Recovery of the radioactivity was greater than 60% of the injected sample. A single peak with the same retention time as 3-Glc was seen; retention of radioactivity as t-Glc and 4-Glc was not detected. EPS

Figure 2. Ability of various metals to induce EPS production in oat protoplasts. Protoplasts were incubated with 200 μM of each compound for 24 h, and the radioactivity incorporated into total ethanol-insoluble material measured ($\pm$ 1 SE (n = 3)).

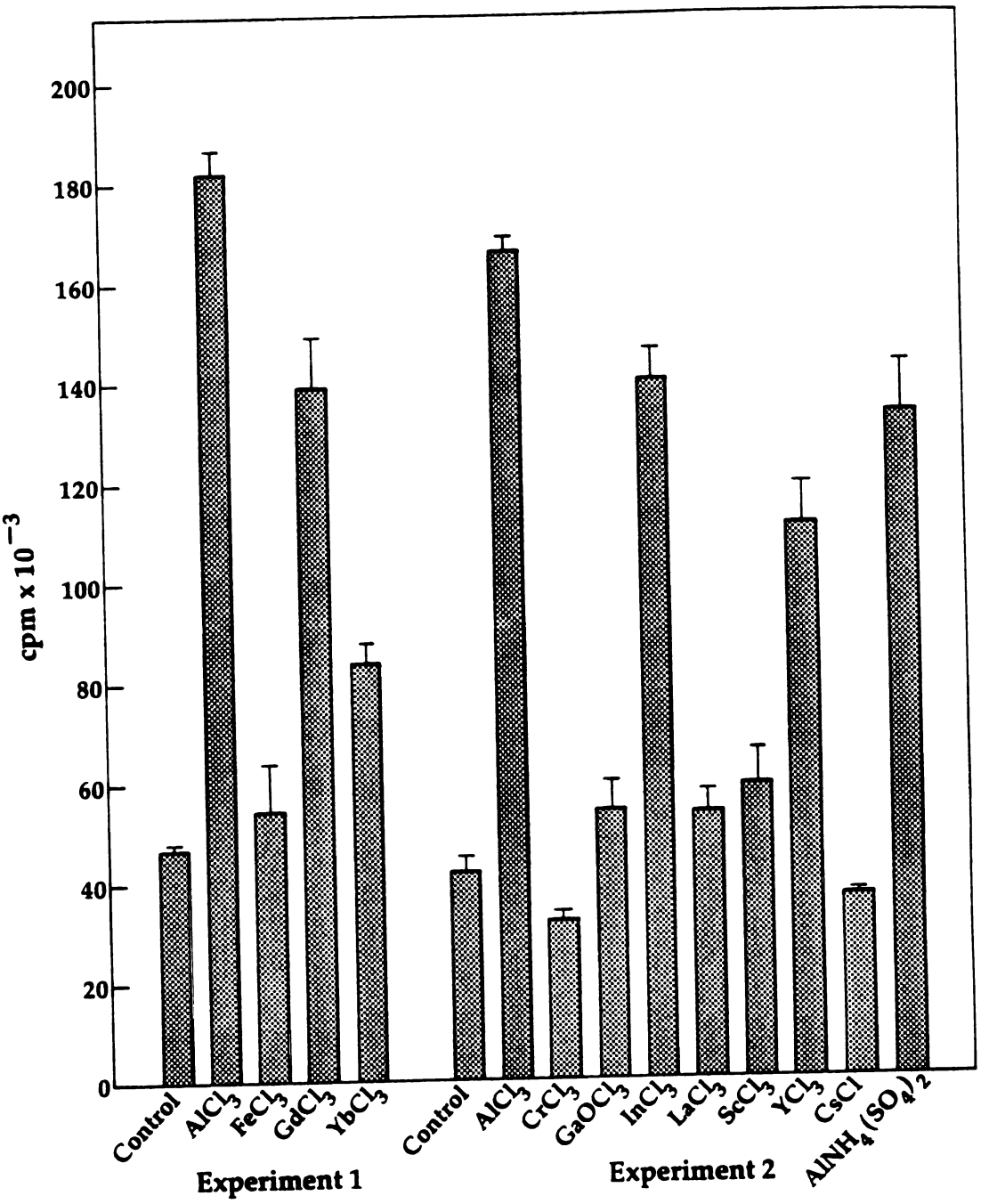
200
180
160
140
120
100
80
60
40
20
0
cpm x 10^{-3}
Control
$AlCl_3$
$FeCl_3$
$GdCl_3$
$YbCl_3$
Experiment 1
Control
$AlCl_3$
$CrCl_3$
$GaOCl_3$
$InCl_3$
$LaCl_3$
$ScCl_3$
YCl_3
$CsCl$
$AlNH_4(SO_4)_2$
Experiment 2

Figure 2

Figure 3. Monosaccharide analysis of EPS. Monosaccharides released by complete acid hydrolysis of EPS produced in 24 h by Park protoplasts were separated by HPLC using an Aminex HPX-87P carbohydrate-analysis column and the effluent was analyzed with an in-line radioactivity detector. The retention times of the standards were determined by refractive index (all sugars) and radioactivity ([^{14}C]Glc and [^{14}C]arabinose). Retention times were corrected for the volume of the tubing between the refractive index and radioactivity detectors. SE's of the retention times of the standards were less than 0.12 min (n = 5). A: control (no treatment); B: treated with 60 pg victorin/ml; C: treated with 200 μM $AlCl_3$.

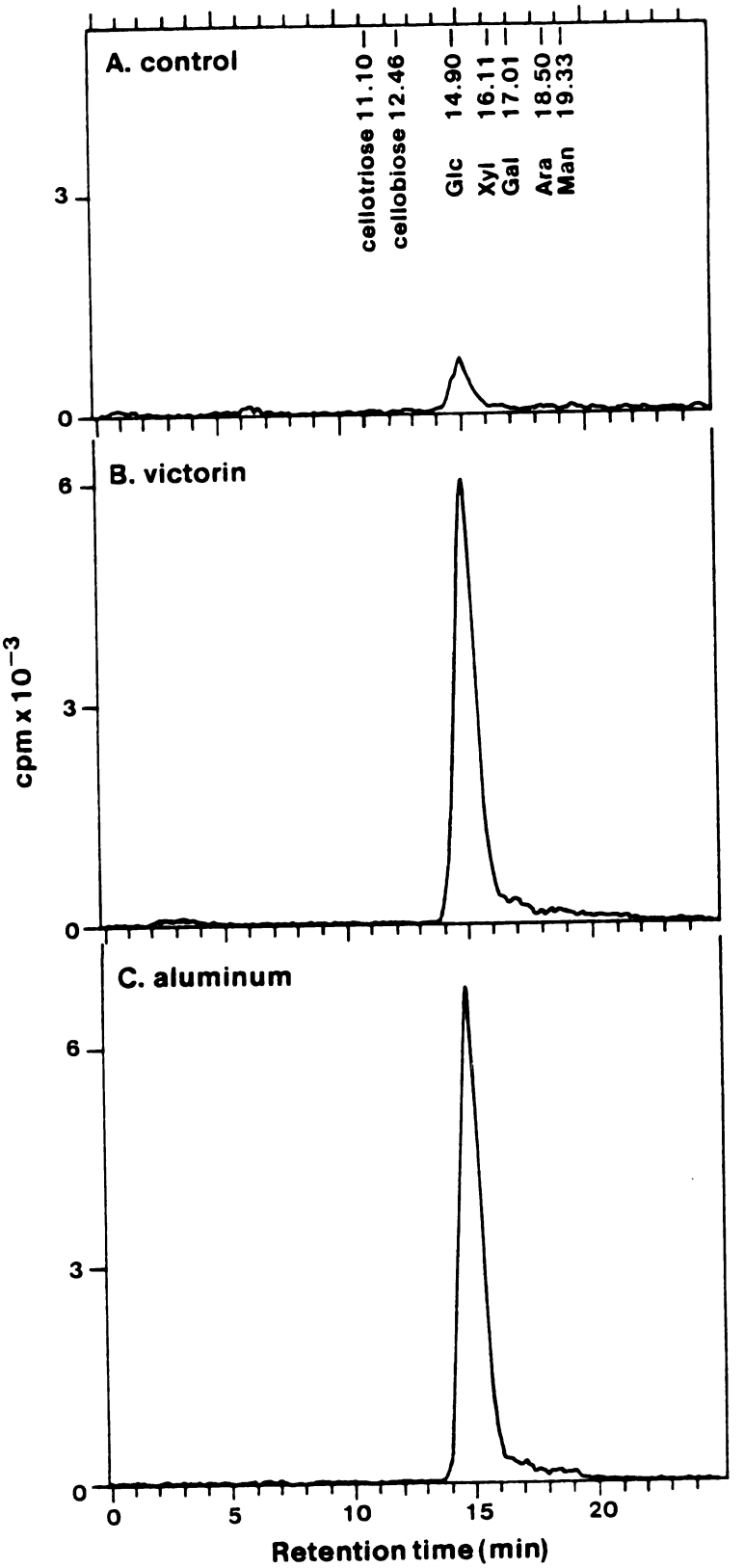
A. control
cellotriose 11.10
cellobiose 12.46
Glc 14.90
Xyl 16.11
Gal 17.01
Ara 18.50
Man 19.33
B. victorin
C. aluminum
cpm x 10⁻³
Retention time (min)

Figure 3

Figure 4. Linkage analysis of EPS. Permethylated alditol acetate derivatives of radiolabelled EPS were hydrolyzed with acid and the products separated by GC. Alditol acetates of nonradioactive methylated laminaribiose (3-Glc) and cellobiose (4-Glc) were added to the sample prior to injection as internal standards. A: internal standards detected by flame ionization; B and C: permethylated alditol acetate sugars derived from EPS (detection by radioactivity); B: sugars of EPS produced by protoplasts in the absence of $AlCl_3$ (control); C: sugars of EPS produced by protoplasts in the presence of 200 μM $AlCl_3$.

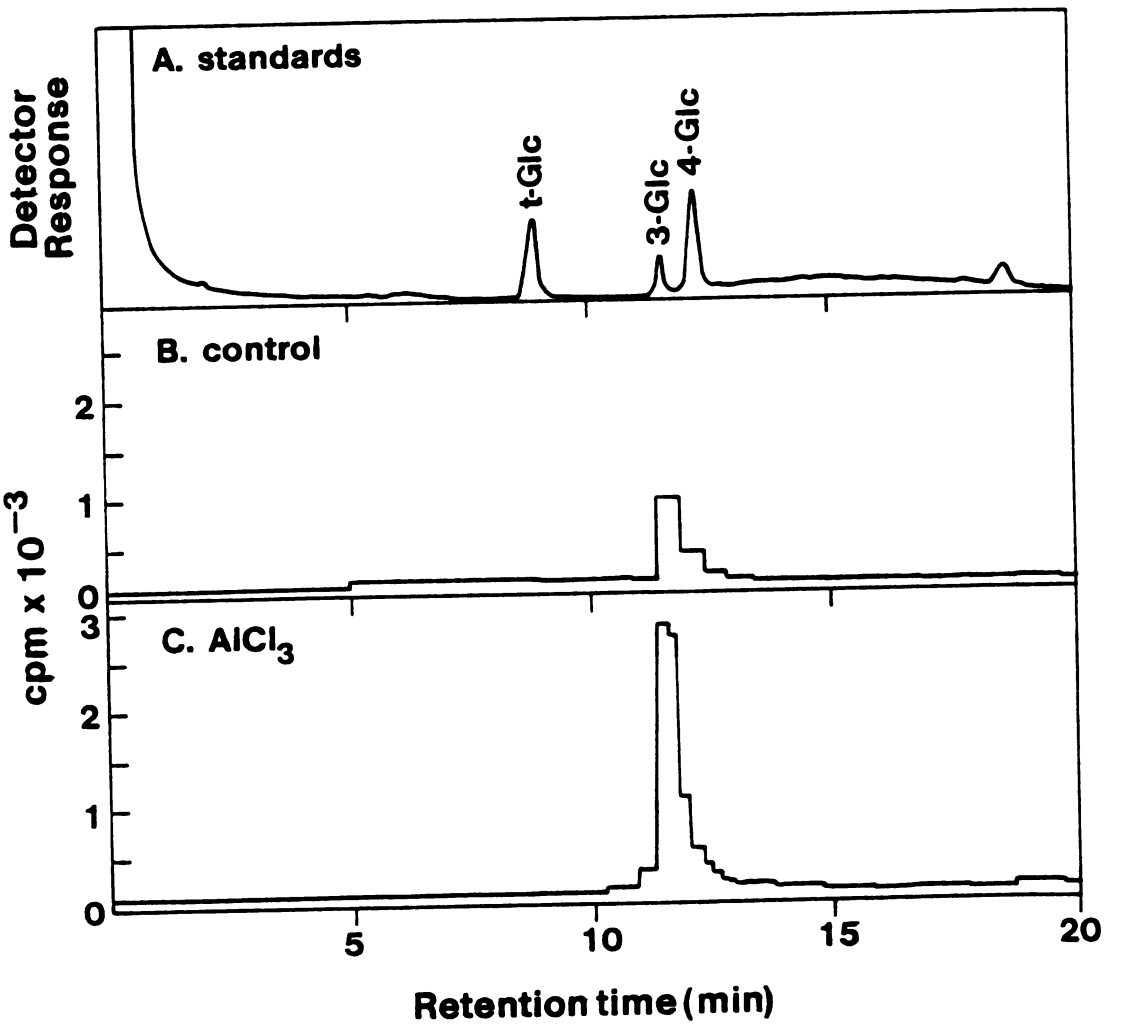
A. standards
t-Glc
3-Glc
4-Glc
Detector Response
B. control
C. AlCl3
cpm x 10-3
0
1
2
3
5
10
15
20
Retention time (min)

Figure 4

induced by victorin (Table 1) and $AlCl_3$ (Table 2) were also degraded by purified *C. carbonum* β (1-3) glucanase. Therefore, EPS is (1-3) β-D-glucan (callose).

Because the permeability of cells is increased by victorin (Wheeler & Black 1963), and also by detergents, perhaps EPS is induced simply by increased membrane permeability. Several detergents were tested for the ability to induce EPS synthesis (Table 3). Of five detergents tested, only digitonin induced EPS production. Therefore, simply increasing membrane permeability does not induce EPS synthesis. It is unclear why digitonin induces EPS synthesis and the others do not, but its effects might not be related to its detergent properties. Digitonin also stimulates callose synthase activity *in vitro* (Hayashi *et al.* 1987; see Appendix 1)

Intracellular Ca^{2+} ions have been proposed to be involved in the action of victorin (Doupnik 1968; Luke & Barnett 1974; Saftner *et al.* 1976) and in the regulation of callose synthase (Kauss 1987). Walton and Earle (1985) observed no changes in EPS production by protoplasts treated with the calmodulin inhibitor trifluperazine or the calcium ionophore A23187. The calcium channel agonists (i.e. Bay K 8644), antagonists (i.e. nifedipine, verapamil, TMB-8), Ca^{2+} chelators (EGTA, BAPTA) and a calmodulin inhibitor that also inhibits callose synthase (chlorpromazine; Bruce P. Wasserman; personal communication) were tested for the ability to induce EPS synthesis in oat protoplasts or to prevent the induction of EPS synthesis caused by victorin. These compounds that interfere with Ca^{2+} metabolism did not affect the EPS induced by victorin and none were capable of inducing EPS synthesis (Table 4).

Table 1. Digestion of victorin-induced [^{14}C]callose by exo-ß1,3-glucanase from Cochliobolus carbonum. Treatments were done in duplicate and are given ± 1 SE. Radiolabelled callose on glass fiber filters was incubated for 24 hr in buffer (50 mM sodium acetate, pH 5.0, plus 0.02% sodium azide) or buffer plus exo-ß1,3-glucanase.

	Protoplast treatment	
	- victorin	+ victorin
	Ethanol-insoluble radioactivity (cpm x 10^{-3})	
Before enzyme incubation	8.4 ± 0.6	72.3 ± 4.6
After incubation in:		
buffer alone	6.6 ± 0.2	57.1 ± 1.7
buffer + glucanase	3.3 ± 0.8	4.0 ± 0.5

Table 2. Digestion of aluminum-induced EPS from oat mesophyll protoplasts with purified laminarinase and pectinase. Ethanol-insoluble material was collected on glass fiber filters after incubation with 200 μM $AlCl_3$ for 24 h, and the radioactivity measured before and after digestion with the enzymes for 6 h at 30°C. "Removed" indicates the difference between before and after enzyme treatment.

Treatment	[^{14}C]Glucose incorporation (cpm x 10^{-3})					
	Control			+200 uM $AlCl_3$		
	Before	After	Removed	Before	After	Removed
laminarinase	4.4	2.0	2.2	24.6	3.3	21.3
pectinase	4.9	4.1	0.8	32.6	28.7	3.9

Table 3: Detergents tested for ability to induce EPS in oat mesophyll protoplasts. Protoplasts were incubated 24 h with concentrations of detergents and EPS production was determined as [^{14}C] Glc incorporation and by visual inspection.

Treatment	Concentrations Tested	[^{14}C]Glc incorporation % of control
digitonin	0.016 %[c]	1100
deoxycholate	1 μM[b]	130
	10 μM[b]	90
	100 μM[a]	10
	1 mM[a]	n.d.
DMSO	0.02 %[b]	110
	0.2 %[b]	100
	2 %[b]	90
	10 %[a]	30
SDS	0.02 %[a]	20
	0.2 %[a]	10
Triton X-100	0.002 %[b]	70
	0.02 %[a]	10
	2 %[a]	

[a] lethal
[b] nonlethal
[c] induces EPS
n.d. not determined

Table 4: Calcium regulating compounds tested for effect on EPS production. Concentration ranges tested are based on previous research (see references).

Compound	Concentrations Tested	[^{14}C]Glc incorporation % of control
Bay K 8644[c]	1 μM	270
	10 μM	140
	100 μM	76
nifedipine[d]	0.1 μM	80
	1 μM	90
	10 μM	100
	100 μM[a]	n.d.
verapamil[d]	1 μM	90
	10 μM	90
	100 μM[a]	90
TMB-8[e]	2 μM	90
	20 μM	110
	200 μM[a]	90
	20 mM[b]	10
+202-791	1 μM[a]	70
-202-791	1 μM[a]	120
BAPTA[f]	10 μM	150
	100 μM[b]	20
	1 mM[b]	10
EGTA[f]	> $[Ca^{2+}]$[b]	n.d.
$LaCl_3$[g]	100 μM	130
	1 mM[a]	150
chlorpromazine	100 μM[a]	100

[a] nonlethal
[b] lethal
[c] Tretyn *et al.* (1990)
[d] Grotha (1986)
[e] Saunders & Jones (1988)
[f] Harrison & Bers (1987)
[g] Martin & Richardson (1979)
n.d. not determined

Protoplasts require Ca^{2+} ions of 1 mM or higher to survive 24 h or longer, probably to maintain membrane structure (Walton & Earle 1985; Marinos 1962). By increasing the victorin concentration to 6 ng/ml, EPS synthesis could be induced in 2 h. When protoplasts were incubated for 2 h in a medium lacking Ca^{2+} in the absence or presence of 6 ng victorin/ml, EPS production was not affected (Table 5).

DISCUSSION

The EPS induced by victorin and $AlCl_3$ described by Walton and Earle (1985) is callose, (1-3)-β-D-glucan. Both Al toxicity and symptoms of Victoria blight resemble calcium deficiency (Foy *et al.* 1978; Godbold *et al.* 1988; Haug & Caldwell 1985; Goodman 1972; Hanchey *et al.* 1968; Saftner *et al.* 1976). Therefore, Ca^{2+} has been hypothesized to be involved in the action of victorin and Al ions on plants. In addition, it has been hypothesized that callose synthase is regulated by Ca^{2+} levels since the enzyme requires Ca^{2+} for enzymatic activity (Kauss 1987). Ca^{2+} may play a role by i) amplitude modulation, i.e. a direct change in Ca^{2+} concentration regulated by fluxes across calcium channels, or ii) by sensitivity modulation, i.e. by increasing the amount of Ca^{2+}-receptor response elements or the affinity of enzymes for Ca^{2+} ions (Hepler & Wayne 1985).

Calcium channels are one way cellular Ca^{2+} levels can be regulated. No calcium channels have been purified from plants but there is evidence that calcium channel agonists and antagonists bind to membranes of plants (Andrejaukas *et al.* 1985; Dolle 1988; Hetherington & Trewavas 1984) and can influence plant responses such as leaf

Table 5: Production of EPS by protoplasts without added calcium in the incubation media. Protoplasts were washed three times with 40 ml containing 0.5 M sorbitol, 40 mM Mes and 50 μM EDTA. The protoplasts were then incubated 2 h $\pm$ 6 ng victorin/ml $\pm$ 10 mM $CaCl_2$. Proteins were removed with Dowex and the radioactivity incorporated into ethanol-insoluble material measured ($\pm$ 1 SE (n=3)).

Treatment	[^{14}C]Glc incorporation $(x10)^{-3}$
-$CaCl_2$	
-victorin	3.3$\pm$0.6
+victorin	8.5$\pm$0.7
+$CaCl_2$	
-victorin	3.3$\pm$0.1
+victorin	10.2$\pm$1.6

movement by *Cassia fasciculata* (Roblin *et al.* 1989) and protoplast swelling in response to red light (Tretyn *et al.* 1990). However, the calcium channel agonists and antagonists tested were unable to mimic or negate the effects of victorin; therefore if calcium is involved it may be by another mechanism or by a type of calcium channel that is unaffected by these chemicals.

Callose is also induced in mesophyll protoplasts of victorin-sensitive oats when treated with trivalent salts of Al, Gd, In, Y, Yb, and the detergent digitonin. There is no obvious correlation between the physico-chemical attributes of the trivalent metals and their ability to induce EPS although Gd, Y, and Al bind to calmodulin (Buccigross *et al.* 1986; Siegal & Haug 1986; Suhayda & Haug 1986; Haug & Caldwell 1985). However, the calmodulin inhibitor trifluoperazine (Walton & Earle 1985) or chlorpromazine (Table 4) did not affect EPS production.

Several chemical treatments tested, $ScCl_3$, $GaOCl_3$ (Fig. 2), deoxycholate, DMSO, Triton X-100, SDS, (Table 3), TMB-8, EGTA, Bay K 8644 (Table 4), nigericin, trifluoperazine, and A23187 (Walton & Earle 1985) were lethal to the protoplasts but did not induce detectable callose formation at sub-lethal concentrations. This suggests that callose production is not a general stress response.

The production of victorin is important for the *C. victoriae* to infect the plant (Scheffer *et al.* 1967). However, the *C. victoriae* must still be able to penetrate the plant. Perhaps the toxin significantly affects the cell before the callose is produced or affects the cells in spite of the presence of the callose.

Another possible role of callose production is to protect against other pathogens. A dominant allele at the *Vb* locus confers susceptibility to *C. victoriae* and also resistance to some races of *Puccinia coronata* (Litzenberger 1949; Welsh *et al.* 1953). Callose production by *C. victoriae*-susceptible oats in response to *P. coronata* has not been described. However, Humphrey and Dufrenoy (1943) observed cell wall changes ("pectic warts") of *P. coronata*-resistant oat cells near necrotic flecks caused by *P. coronata*; however, the composition was not analyzed. It would be interesting to know if these "pectic warts" are in fact callose depositions induced by attempted infection by both *P. coronata* and *C. victoriae* on this genotype of oats and if so, determine if it is a successful barrier to *P. coronata*.

CHAPTER 4

EPS induction by Al ions in tolerant and sensitive varieties of monocots

ABSTRACT

Mesophyll protoplasts of oat (*Avena sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) produce an extracellular polysaccharide (EPS) in response to concentrations of $AlCl_3$ greater than 10 μM. Mesophyll protoplasts from the acid-soil tolerant oat cultivar Coker 83-23 produced less EPS in response to $AlCl_3$ than the acid-soil sensitive cultivar Fla 501 and the Al-tolerant cultivar of wheat Atlas produced less EPS than the Al-sensitive cultivar Scout. However, the Al-tolerant cultivar of barley Dayton produced more EPS than the Al-sensitive cultivar Kearney. No consistent relationship between EPS production and acid-soil tolerance was identified within near-isogenic lines of wheat. Therefore, production of EPS by mesophyll protoplasts in response to Al ions does not appear to be related to Al ion tolerance in whole plants of these varieties.

INTRODUCTION

Aluminum, the most abundant metal on the earth's surface, was first implicated in toxicity to plants in 1918 (Hartwell & Pember). Interest in the phytotoxicity of Al has increased recently due to the hypothesized role in plant decline in areas affected by acid precipitation, brought about by the increase in the solubility of Al in soils (Cronan & Schofield 1979; Godbold *et al.* 1988; Haug 1984). Although the mechanism of Al toxicity is not well understood, some responses are well documented. Al ions inhibit root elongation, produce symptoms of nutrient deficiency, particularly of calcium, and increase drought susceptibility (Foy *et al.* 1978; Godbold *et al.* 1988; Hartwell & Pember 1918). Al ions cause rapid changes in the ultrastructure of the Golgi apparatus of roots (Bennet *et al.* 1985). Al ions also inhibit the binding of Ca^{2+} to calmodulin, a Ca^{2+}-binding protein (Siegal & Haug 1983), as well as promoting tubulin assembly *in vitro* (MacDonald *et al.* 1987). Physiological changes have also been observed. Bennet *et al.* (1985) have observed increased mucilage or root cap slime production in roots of maize after exposure to Al ions. Horst *et al.* (1982) have observed that callose is induced in pea roots in response to 93 μM $AlCl_3$.

Studies of Al toxicity are complicated not only by changes in it's

solubility with changes in pH but also because Al ions can exist as different species in solution. In fact, plants may respond to only a subset of the total Al ions present. The ratios of these different species depend on the pH and presence of other ions within the solution. A thorough review of Al speciation in soils has been written by Lindsay (1979). In brief summary, following the addition of Al salts to soils, Al ions may exist as several oxide and hydroxide minerals. The presence of Al^{3+} ions in equilibrium with these minerals is pH-dependent. Moreover, Al^{3+} ions do not exist freely in solution but are surrounded by six H_2O molecules. With increased pH, protons are released from this complex resulting in a series of hydrolysis products which also exist in equilibrium. Therefore, the addition of Al salts to a plant does not result in a homogeneous population of Al ions but rather a mixture of species. For this reason, experimental conditions must be carefully designed because the plant presumably responds to the concentration of the toxic species rather than the total concentration of Al ions present. Currently, it is thought that Al^{3+} species is responsible for the toxicity in acidic soils (Kinraide & Parker 1987). However, there is some evidence that other species i.e. mononuclear hydroxy-Al hydrolysis products (Kinraide & Parker 1989) may be toxic.

Both Al ions and victorin (the host-selective phytotoxin produced by the fungal pathogen, *Cochliobolus victoriae*) cause mesophyll protoplasts of oat (*Avena sativa* cv Park) to produce callose (Schaeffer & Walton 1990; Chapter 3). Callose induction by victorin only occurs in protoplasts of plants susceptible to *C. victoriae*. In addition, a number of studies have shown that both Al

(Bennet *et al.* 1985) and victorin (Hanchey 1980) cause alterations, including apparent stimulation of deposition by the Golgi apparatus, where all non-cellulosic cell wall polysaccharides are thought to be synthesized (Fincher & Stone 1981). Symptoms of both Al toxicity and Victoria blight resemble Ca^{2+} deficiency and symptoms can be diminished by the addition of Ca^{2+}. Both victorin and Al ions cause inhibition of root elongation which has been frequently used as a bioassay of these toxic effects. In contrast to victorin, which is active at 75 pM, Al ions are active only at concentrations above 10 μM. However, as described earlier, the actual concentration of the toxic species of Al ions may be considerably lower.

Since callose induction in oat mesophyll protoplasts in response to victorin is related to the sensitivity of the plant to victorin (Walton & Earle 1985), we were interested in determining if Al ions and victorin act by a similar mechanism. Mesophyll protoplasts of *A. sativa* cv. Park produce callose in response to Al ions. Oat varieties known to differ in Al tolerance and other plant species were tested for the ability to produce an extracellular polysaccharide (EPS) in response to $AlCl_3$, with particular interest in determining if EPS production is related to Al tolerance. Portions of this work have been published (Schaeffer & Walton 1990).

MATERIALS AND METHODS

Plant Varieties and Growth Conditions. Oat (*Avena sativa* L.) cultivars used were cv Park (sensitive to victorin), cv Old Fulgrain (insensitive to victorin), cv

Coker 83-23 (tolerant to acid soil), and cv Fla 501 (sensitive to acid soil) (Foy *et al.* 1987). Barley (*Hordeum vulgare*) cultivars used were cv Dayton (aluminum-tolerant) and cv Kearney (aluminum-sensitive) (e.g. Reid *et al.* 1969). Wheat (*Triticum aestivum*) cultivars used were cv Atlas 66 (aluminum-tolerant) and cv Scout 66 (aluminum-sensitive) (e.g. Foy *et al.* 1965; Foy *et al.* 1967). None of these cultivars were near-isogenic. The oat cvs Coker 83-23 and Fla 501, the barley cvs Dayton and Kearney, and wheat cvs Atlas 66 and Scout 66 were obtained from Dr. Charles Foy, Plant Stress Laboratory, Plant Physiology Institute, ARS, USDA, Beltsville, MD. Oat cv Park and cv Old Fulgrain were obtained from Dr. Jonathan Walton, Michigan State University.

Near-isogenic lines of wheat were the generous gift of Dr. Emmanuel Delhaize, CSIRO, Canberra, Australia. These lines were developed by Fisher and Scott (1987) by crossing Carazinho (Al-tolerant line from Brazil containing a major dominant allele which segregates in a Mendelian manner for Al tolerance) and Egret (Al sensitive line from Australia). The progeny of this cross were backcrossed three times to Egret or Egret derivatives. Therefore, these lines are at most 88% isogenic. Eight of these F_3 lines were selected for tolerant and sensitive members. The phenotypes of these progeny with respect to hematoxylin staining and root growth inhibition were the same as the parent (Fisher & Scott 1987). Wheat cultivars Warigal (Al-sensitive) and Waalt (Al-tolerant selection of Warigal) were also a gift from Dr. Emmanuel Delhaize, CSIRO, Canberra, Australia and have been previously described (Larkin 1987).

Seeds were planted in soil composed of equal parts of peat moss,

vermiculite and perlite, and grown in a growth chamber under fluorescent and incandescent lamps (fluence rate of photosynthetically active radiation: 140 μmol/m^2·sec) for a 12 h photoperiod at 18°C for 20 to 30 days. In experiments comparing two cultivars, the plants were always of the same age and protoplasts from each were prepared simultaneously.

Protoplast preparation. Sterile protoplasts were prepared by methods modified from Walton and Earle (1985). Leaves (500 mg) were abraded with carborundum and incubated at 26°C for 3 h in 2% (w/v) Cellulysin in SCM (0.5 M sorbitol, 10 mM $CaCl_2$, and 40 mM Mes), pH 5.5. Following release, protoplasts were filtered through 80-μm nylon screen mesh, washed twice with SCM, pH 6.0, and collected by centrifugation at 100 *g*. The protoplasts were resuspended in SCM, pH 6.0, at a final concentration of 40,000 protoplasts/ml, and dispensed at 0.5 ml/well into 24-well Falcon multiwell plates (Becton-Dickinson). Since Al ions are often present in standard laboratory water and can be released from glassware (Sternweis & Gilman 1982), SCM was analyzed for Al ion content by atomic absorption spectroscopy (W. Berti, Department of Crop and Soil Sciences, Michigan State University); SCM medium contained 8 μM Al ions.

Microscopy. After 24 h incubation, protoplasts were visually observed for EPS production by staining with a 1:1 mixture of 0.1% Calcofluor white in SCM, pH 6.0, or 0.2% Sirofluor in 2% K_3PO_4 and viewing under a Zeiss epifluorescence

microscope (filter set G 365) at a final magnification of 400x. Photographs were taken with Kodak Plus-X film. Protoplast survival was evaluated by exclusion of Evan's blue from the cytoplasm.

[^{14}C]Glucose Incorporation. Immediately after dispensing protoplasts into the multiwell plates, $AlCl_3$ and 0.25 μCi sterile D-[U-^{14}C]Glc in SCM, pH 6.0, was added to each well. The plates were sealed with parafilm and incubated at room temperature (22°C) in darkness for 24 h. The protoplasts were then transferred to 15-ml test tubes. The wells were washed twice with 1.0 ml of 80 mM citric acid pH 3.0, and the material was transferred to the same tube. The decrease in osmotic pressure by addition of 80 mM citric acid caused the protoplasts to burst allowing the cellular debris to be separated from the EPS.

To remove proteins, 1.0 ml of suspended Dowex 50W cation exchange resin equilibrated with 80 mM citric acid, pH 3.0, was added to each tube containing the protoplasts and two washes. The tubes were rocked for 2 h and, after allowing the resin to settle, the supernatants were transferred to 20-ml tubes with one 1.0 ml (80 mM citric acid, pH 3.0) wash of the resin. After addition of ethanol to a final concentration of 70% (v/v, assuming additive volumes), the samples were stored overnight at 4°C, and then filtered through Whatman GF/A glass fiber filters using a Millipore model 1225 sampling manifold. The filters were rinsed twice with cold 70% ethanol and the retained radioactivity measured in a scintillation counter.

Chemicals. $AlCl_3$ and Dowex 50W cation exchange resin (100-200 mesh, 2% crosslinkage) were obtained from Sigma. D-[U-^{14}C]Glc, 258.5 mCi/mmol, was from NEN. Sirofluor was a gift from Deborah Delmer, Hebrew University, Jerusalem, Israel. Calcofluor white from American Cyanamid used in previous studies (Walton & Earle 1985) was used for fluorescence microscopy; "Fluorescent Brightener 28" - F 6259 from Sigma resulted in poor fluorescence of EPS.

RESULTS

Based on observation by fluorescence microscopy, maximal callose production was induced in oat mesophyll protoplasts after 24 h treatment with 100 to 200 μM $AlCl_3$. After 24 h in 100 μM $AlCl_3$, 62% of the Park and 79% of the Old Fulgrain protoplasts survived. At 1 mM $AlCl_3$, fewer than 2% of the protoplasts survived and for this reason less callose was visible. Oat protoplasts incubated in SCM medium, pH 6.0, plus [^{14}C]Glc incorporated radioactivity into callose. Incorporation of [^{14}C]Glc into callose was stimulated 10 to 20-fold over controls in the presence of 100 μM $AlCl_3$ (Fig. 1).

In four separate trials, protoplasts from oat cv Old Fulgrain (victorin-tolerant) consistently produced 20 to 30% more callose than protoplasts of cv Park (victorin-sensitive) in 100 μM to 1 mM $AlCl_3$ (Fig. 1). To test whether such cultivar differences could be related to differential Al sensitivity, oats known to differ in tolerance to high-Al acidic soils were compared (Foy, *et al.*,

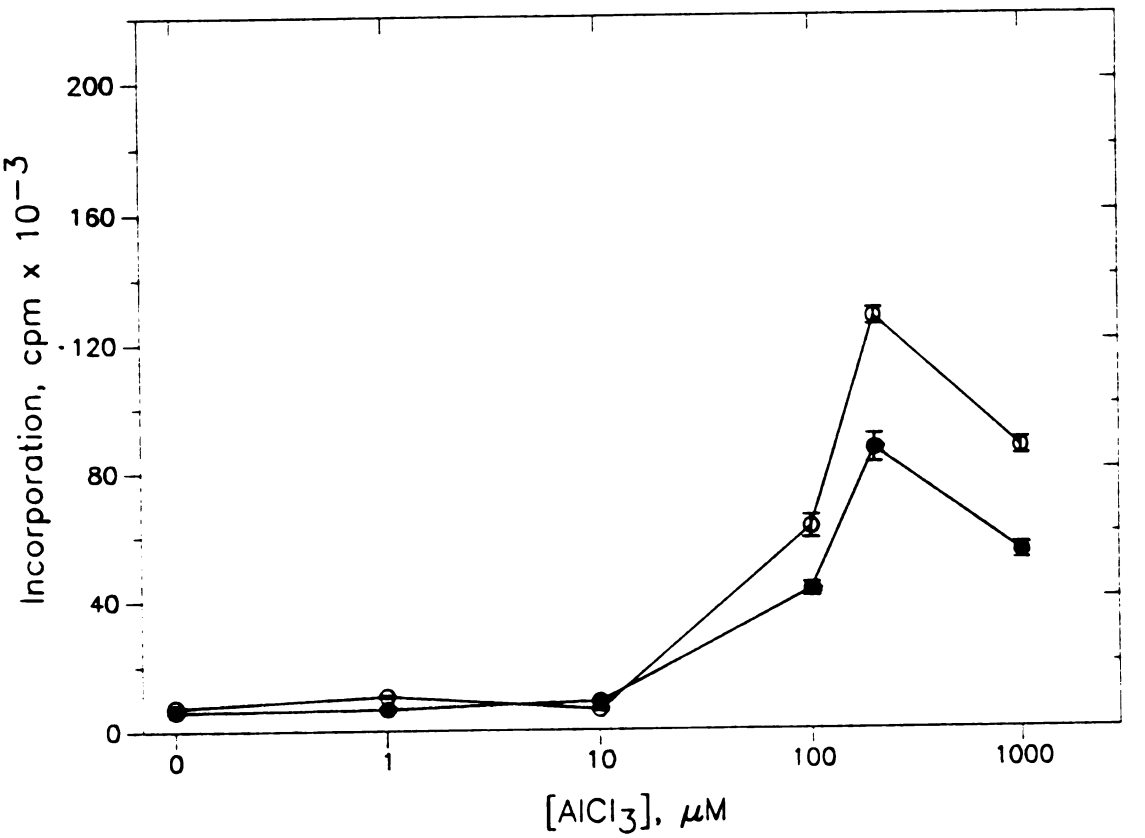

Figure 1. Incorporation of [^{14}C]Glc into EPS by oat mesophyll protoplasts as a function of $AlCl_3$ concentration. Protoplasts (20,000/well) were incubated for 24 h with 0.25 μCi [^{14}C]Glc plus $AlCl_3$ at different concentrations. Callose (insoluble in 70% ethanol) was collected by filtration. Oat cv Old Fulgrain O; cv Park ●. Error bars represent $\pm$ 1 SE (n = 3).

1987). Oat cv Fla 501 (sensitive to acid soil) consistently produced more EPS as judged microscopically, and, at 200 μM $AlCl_3$, produced 35 to 40% more EPS than cv Coker 83-23 (tolerant of acid soil) on the basis of [^{14}C]Glc incorporation (Fig. 2A).

Mesophyll protoplasts from Al-tolerant and Al-sensitive pairs of barley and wheat cultivars were also tested for EPS induction in response to $AlCl_3$. All produced EPS in response to Al ions, and all were killed by 1 mM $AlCl_3$. Similar to oats, the Al-sensitive wheat cv Scout 66 produced more EPS than the Al-tolerant cv Atlas 66 (Fig. 2C). In contrast, the Al-tolerant cultivar of barley Dayton in 200 μM $AlCl_3$ produced more EPS than the Al-sensitive cv Kearney (Fig. 2B).

Four near-isogenic lines and two related lines of wheat differing at a single gene for Al tolerance were also tested for a relationship between Al tolerance and EPS induction (Fig. 3). In line C the tolerant variety produced more EPS than the sensitive variety in the presence of greater than 100 μM $AlCl_3$. However, in the other lines derived from the same parents, there was no consistent correlation between Al tolerance and EPS production within replicate experiments of the same lines or between lines.

Studies of Al phytotoxicity are confounded by the ability of Al ions to exist as numerous chemical species, depending on the pH of the solution. Al becomes more soluble, and the concentration of Al^{3+} as a percentage of total Al increases, with decreasing pH (Kinraide & Parker 1989). The concentration of Al^{3+} as a percentage of total Al is very low at pH 6.0, the pH at which

Figure 2. Effect of $AlCl_3$ concentration on EPS production by cultivars of oat, barley and wheat. Mesophyll protoplasts of each cultivar were incubated for 24 h with 0.25μCi [^{14}C]Glc. Protein was removed with cation exchange resin at pH 3.0 and the EPS was precipitated with ethanol and collected by filtration. Al-sensitive cultivars: ○; Al-tolerant cultivars: ●. Error bars represent $\pm$ 1 SE (n = 3).

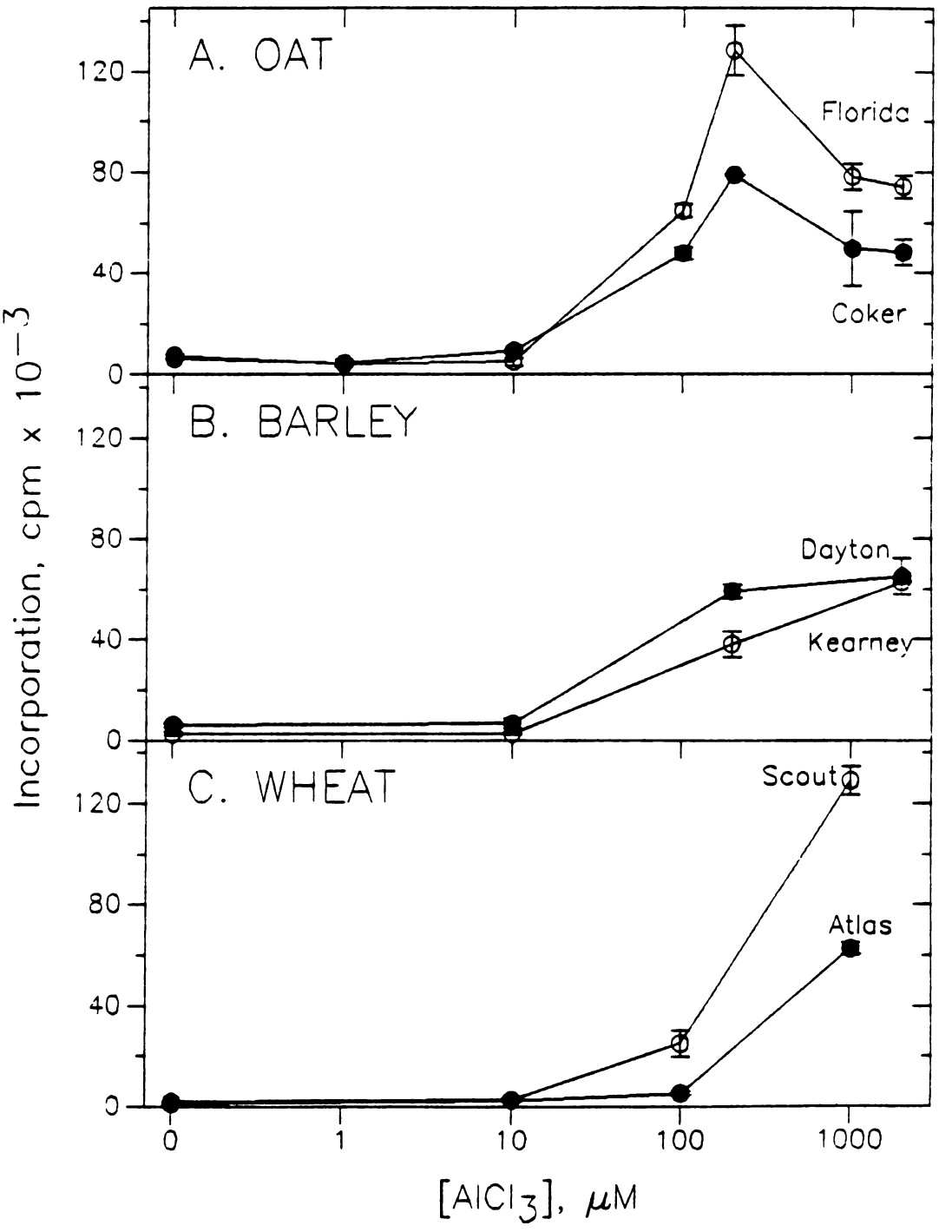
A. OAT
Florida
Coker
B. BARLEY
Dayton
Kearney
C. WHEAT
Scout
Atlas
120
80
40
0
0
1
10
100
1000
Incorporation, cpm x 10^{-3}
[$AlCl_3$], μM

Figure 2

Figure 3. EPS production by near-isogenic lines of wheat. Al tolerant and sensitive pairs of four near-isogenic and two related wheat lines were compared for EPS production as a function of $AlCl_3$ concentration. Each graph contains data from two experiments; closed symbols = experiment 1; open symbols = experiment 2. □ = tolerant; Δ = sensitive. Standard error was less than 10% (n = 3).

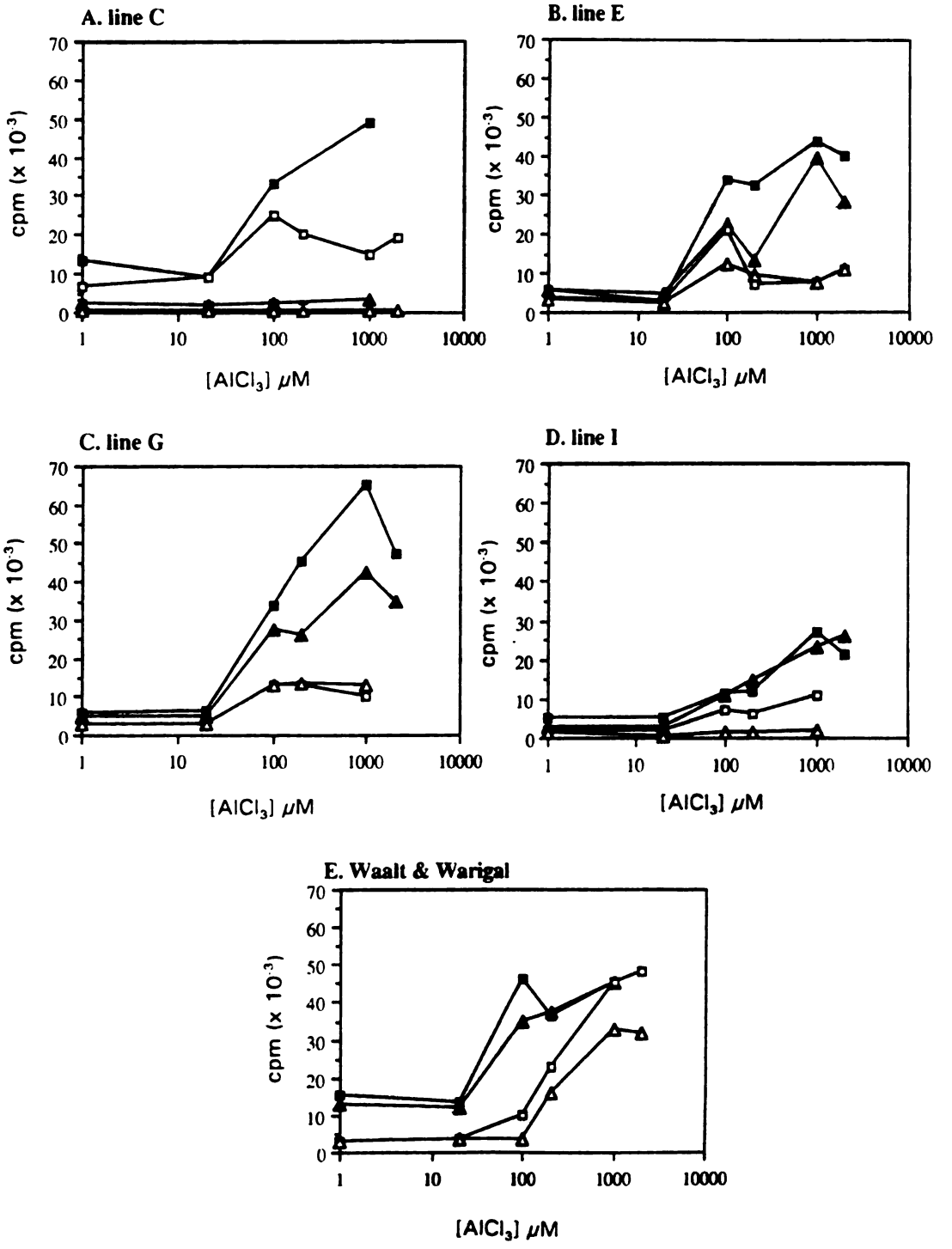
A. line C
B. line E
C. line G
D. line I
E. Waalt & Warigal
cpm (x 10⁻³)
[AlCl₃] μM
0
10
20
30
40
50
60
70
1
10
100
1000
10000

Figure 3

these experiments were done. Therefore, if Al^{3+} is the active Al species (Kinraide & Parker 1987), callose induction should become more sensitive to $AlCl_3$ as the pH is decreased. Attempts to study Al -induced EPS production in unbuffered solutions adjusted to pH 4.0, 5.0, and 6.0 were unsuccessful because after 24 h incubation the pH values of the protoplast solutions were 4.0 to 4.3, presumably due to the metabolic activity of the protoplasts. Acetate buffer (pK 4.8) was toxic to the protoplasts, and succinate buffer (pK_1 4.2, pK_2 5.6) prevented callose production perhaps by complexing Al ions, similar to other dicarboxylic organic acids (Haug 1984; Suhayda & Haug 1987). Protoplasts buffered with 40 mM Hepps (pK_1 3.8, pK_2 7.8: N. Good; personal communication) at pH 4.0 survived but produced no callose in response to 10, 30, or 100 μM $AlCl_3$. In the presence of 100 μM $AlCl_3$, protoplasts produced 50% more callose in SCM pH 5.0 than at pH 6.0. However, at pH 5.0, in either SCM or SCM with Hepps in place of Mes, protoplasts produced callose at 100 μM $AlCl_3$ but not at 10 or 30 μM $AlCl_3$. Hence, decreasing the pH from 6.0 to 5.0 caused an increase in EPS production but not an increase in sensitivity to $AlCl_3$.

DISCUSSION

Callose (β (1-3) glucan) is produced by plant cells in response to a variety of mechanical, pathological, and chemical stresses, including exposure to heavy metals (Fincher & Stone 1981) and exposure to other metals such as Al (Horst

et al. 1982; Schaeffer & Walton 1990; Chapter 3). The data presented here show that Al also induces EPS production, presumably callose, by mesophyll protoplasts of oats, barley, and wheat. Maximal induction occurred at 200 μM $AlCl_3$ at pH 6.0 after 24 h incubation (Fig. 1,2). At higher Al concentrations the protoplasts collapse within 24 h but still synthesize some EPS.

Root cap slime has been implicated in the protection against Al ions in roots of *Vigna unguiculata* (Horst *et al.* 1982), and roots of the Al-tolerant barley cv Dayton produce more root cap slime than the Al-sensitive cv Kearney (Hecht-Buchholtz & Foy 1981). Callose is clearly different from root cap slime, since it is produced by mesophyll cells and contains only glucose (Schaeffer & Walton 1990; Chapter 3) whereas root cap slime also contains galactose, fucose, and uronic acids (Harris & Northcote 1970).

To test the possibility that the response of whole plants to Al ions might be related to the induction of EPS production by mesophyll protoplasts, Al-tolerant and Al-sensitive cultivars of oat, barley, and wheat cultivars were compared in parallel (Fig. 2). In near-isogenic lines of wheat differing in Al ion tolerance, there are differences in magnitude and reproducibility of EPS production to Al ions; therefore EPS production to Al ions is not related to Al tolerance within or between these lines (Fig. 3).

The conclusions from these experiments are several. First, Al ions induce EPS not only in victorin-sensitive oats but also in wheat, barley, and other oat varieties. Second, although there are reproducible differences in EPS production between the unrelated pairs of Al-sensitive and Al-tolerant cultivars

of wheat barley and oats, they are rather small in comparison with the differences between these same cultivars in Al sensitivity at the whole plant level (Foy *et al.* 1965; Foy *et al.* 1967; Foy *et al.* 1987). Sometimes Al tolerant varieties make more EPS and other times the Al sensitive varieties make more EPS. Third, there is no correlation between EPS production and Al tolerance in near-isogenic lines of wheat. Al ions tend to accumulate in roots with little Al transported to other plant parts in wheat and barley (Foy *et al.* 1967), therefore the response of leaf protoplasts of these species may not be related to the plant's response. The overall conclusion is that EPS production by mesophyll protoplasts in response to Al ions is probably not related to whole plant sensitivity to Al.

CHAPTER 5

FUTURE DIRECTIONS

FUTURE DIRECTIONS

An exo β (1-3) glucanase has been cloned from the plant pathogenic fungus, *C. carbonum*. This enzyme is not necessary for pathogenicity of maize. However, it might well be important in other disease interactions. Therefore, the *C. carbonum EXG1* clone could be used to isolate and characterize β (1-3) glucanases of other pathogens. In addition, because the gene product of *EXG1* degrades callose and because callose production by some plants is correlated with resistance of these plants to pathogens (i.e. Bonhoff *et al.* 1987; Hinch & Clarke 1982; Bayles *et al.* 1990; also see Chapter 1), the ability to degrade callose by other pathogens could improve their ability to infect otherwise resistant hosts. Is callose alone responsible for blocking the pathogen's progression into host tissue? If these pathogens were transformed with the *EXG1* gene of *C. carbonum* would they become better pathogens?

The role of other carbohydrate-degrading enzymes in plant-pathogen interactions is also of interest. For example, two other β (1-3) glucanases have been identified in *C. carbonum*. Are these important to disease progression? In addition, these enzymes coelute with mixed-linkage glucanase activity. One question to address is whether these mixed-linkage glucanases are capable of degrading both mixed-linkage glucans and β (1-3) glucans. Is one enzyme

capable of degrading both substrates or are these activities the result of two different enzymes? Since mixed-linkage glucans are present in monocot cell walls, mixed-linkage glucanases may be important to plant-pathogen interactions. Studies of this nature are possible using approaches similar to those in Chapter 2.

Callose is produced by mesophyll protoplasts of *C. victoriae*-susceptible oats in response to victorin (Chapter 3). Since the plants are successfully infected by this pathogen, presumably callose is not a successful defense against *C. victoriae* or victorin. However, these plants are resistant to some races of *Puccinia coronata* (Litzenberger 1949; Welsh *et al.* 1953). Is callose biosynthesis induced by *P. coronata*? The descriptions by Humphrey and Dufrenoy (1944) indicate that some ultrastructural changes occur. If these "pectic warts" that they describe fluoresce in the presence of Sirofluor, then perhaps they contain callose. However, this would be difficult to determine *in planta* because Sirofluor also stains mixed-linkage glucans (Evans & Stone 1984) which are a component of oat cell walls (Stinard *et al.* 1980). Another way to address this question is to determine if callose is produced by oat protoplasts in response to germinating *P. coronata* spores (similar to the analysis in Chapter 3).

It is unknown if *P. coronata* produces a compound similar to victorin. If one is produced it may either be a component of the fungus or a secreted molecule. Studies of *P.coronata* are challenging because *P. coronata* is an obligate pathogen, unable to grow in the absence of the plant. The fungus may

be grown on the plant, however, and then the infected plant analyzed for compounds capable of inducing callose which are not present in the uninfected plant. Any molecules unique to the infected plant which induce callose are candidates for similarities to victorin and may be purified for structural analysis. In addition, victorin-specific antibodies may react to epitopes of a *P. coronata* callose- inducer if there are similarities.

By combining biochemical studies of the enzyme and its action with physiological studies of how callose synthase is regulated with callose induction in the plant a more complete understanding will be possible. For example, microsomes of protoplasts can be analyzed for the quantity of callose synthase activity (Appendix 1). This protoplast system may be useful for analyzing how callose synthase is induced. Almost ten-fold more callose is produced by victorin-treated protoplasts than untreated protoplasts. However, there is less than a two-fold increase in the amount of callose synthase present in the microsomes of these protoplasts. In addition, isolation of microsomes from noncallose-producing tissue results in high levels of callose synthase activity. Why is callose synthase activated during biochemical extraction? By inhibiting the activation of callose synthase during isolation of microsomes, greater differences in callose synthase activity between induced and uninduced samples may result (see Appendix 1 for more discussion).

Several difficulties exist with analyzing callose synthase which need to be addressed. i) Microsomes of different plants and different tissues of the same plant contain different microsomal amounts of callose synthase activities

(see Appendix 1; results). Ideally, very active microsomes are desirable but only if callose can be induced in the original tissue. ii) There appear to be plant specific differences in callose inducers, i.e. nifedipine and verapamil (Grotha 1986; Chapter 3). iii) Differences in callose synthase effectors are described in the literature (Kauss *et al.* 1983; Sloan & Wasserman 1989; see Appendix 1 for further discussion). Therefore, work of different plant systems cannot be directly compared.

The role of Ca^{2+} ions in victorin action and callose synthase is not yet understood. Based on the results in Chapter 3, Ca^{2+} ions do not appear to be involved in stimulation of callose biosynthesis. Perhaps, instead, Ca^{2+} ions are important because they regulate other enzymes within the cell. Research by Delmer *et al.* (1991) suggests that the regulation of callose synthase may be quite complex. In addition, Ca^{2+} ions are important as second messengers and in various aspects of signalling pathways including phosphorylation and phosphatidyl inositide metabolism (Hepler & Wayne 1985). Therefore, Ca^{2+} ions may not affect callose synthesis or victorin action except indirectly.

Further studies of the callose induction by plants and its degradation by pathogens will indicate those interactions where callose serves as a defense against pathogens.

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APPENDIX 1

The regulation of β (1-3) glucan synthase during callose induction by victorin

ABSTRACT

Increased callose production by mesophyll protoplasts of *Avena sativa* is detectable after 2 h treatment with 6 ng victorin/ml. The production of callose by victorin-treated protoplasts is 4 to 6-fold greater than that produced by untreated protoplasts. Microsomes isolated from these protoplasts contain 50% more callose synthase activity than those from untreated protoplasts. Microsomal callose synthase activity is lower in the absence of Ca^{2+} ions or in the presence of EDTA. Addition of victorin to the microsomes does not significantly affect the callose synthase activity.

INTRODUCTION

Callose, a β (1-3) glucan, has been observed in plants as a component of pollen tubes (Rae *et al.* 1985), sieve plates (Eschrich & Currier 1964), and phloem plugs (Mangin 1889) but it is also produced by plants in response to biotic and abiotic stresses including pathogen attack (Aist 1976), mechanical wounding (Eschrich 1975; Brett 1978; Currier 1957), and heat (Majunder & Leopold 1967; McNairn 72). Callose may be deposited within the cell wall at sites of mechanical damage or as a component of papillae, as, for example, in the papillae of maize roots in response to *Phytophthora cinnamomi* (Hinch & Clarke 1982).

The role of callose is presumably to isolate the infected or wounded area from the rest of the plant, thereby retarding pathogen invasion (Aist 1976). Maize, a nonhost, produces callose in response to *Phytophthora cinnamomi* which is unable to significantly invade the plant. However, the host plant *Lupinus angustifolius* does not produce callose during infection and invasion is successful (Hinch & Clarke 1982). In addition, callose is produced in roots of resistant but not susceptible *Glycine max* in response to attempted infection by *Phytophthora megasperma* f. sp. *glycinea* (Bonhoff *et al.* 1987). Moreover, decreasing the frequency of callose-containing papillae by treating barley with

2-deoxy-glucose resulted in increased infection by *Erisyphe graminis* (Bayles *et al.* 1990).

Callose is also a stress response. For example, callose is produced by oat protoplasts in response to victorin and $AlCl_3$, which are toxic to protoplasts (Chapter 1; Schaeffer & Walton 1990). In addition, inhibiting callose formation with either EGTA or 2-deoxy-glucose in *Phaseolus vulgaris* (a nonhost) did not increase the frequency of haustorium formation by *Uromyces vignae* (Perumalla & Heath 1989). One explanation may be that callose can block and therefore protect only against some inducers. Other callose inducers may be able to lethally damage the cell either before the callose is produced or in spite of the production of callose. As a further complication, not all plants produce callose in response to the same conditions. For example, nifedipine and verapamil induce callose in *Riella helicophylla* (aquatic liverwort)(Grotha 1986) but not in oats (Chapter 1). In addition, Kauss *et al.* (1983) have reported that proteases increase callose activity in suspension cultured soybean cells although Sloan and Wasserman (1989) observe a decrease in activity after protease treatment of red beet membranes. This suggests that there may be some species-specific differences in the role of callose as well as differences in the mechanism of callose induction and regulation.

The enzyme responsible for callose biosynthesis, 1,3 β D-glucan synthase (E.C. 2.4.1.34; also called callose synthase) is active in membrane preparations from a variety of plants and tissues although callose is only a minor component in cell walls. In fact, callose synthase activity was first observed in membranes

of tissue during efforts to study cellulose biosynthesis (Delmer 1983). However, when wounded, plants rapidly produce callose at the sites of mechanical or pathogen damage suggesting that the regulation of callose synthase is extremely effective. It has been hypothesized that cellulose synthase and callose synthase are the same enzyme (Jacob & Northcote 1985; Delmer 1987a). In response to perturbations, it is proposed that the enzyme is changed from one producing (1-4)-linked glucose (cellulose) to one producing (1-3)-linked glucose (callose) using the same substrate, UDP-Glc (Brett, 1978; Péaud-Lenoël & Axelos 1970).

Schmele and Kauss (1990) showed that the amount of callose synthase activity is increased in the microsomes of cucumber induced for systemic resistance to *Colletotrichum lagenarium* by first infecting with *Colletotrichum lagenarium* or tobacco necrosis virus. This increased microsomal enzyme activity was correlated with observed callose production *in vivo*. In this study, we were interested in determining whether or not the callose synthase activity of microsomes from oat protoplasts increased after victorin treatment. In this system, the quantity of victorin-induced callose production by the protoplasts can be compared to the callose synthase activity of the microsomes.

MATERIALS AND METHODS

Plant Varieties and Growth Conditions. Seeds of oat (*Avena sativa* L.) cv Park (sensitive to victorin), were planted in soil composed of equal parts of peat

moss, vermiculite, and perlite and grown in a growth chamber under fluorescent and incandescent lamps (fluence rate of photosynthetically active radiation: 140 μmol/m^2·sec) for a 12 h photoperiod at 18°C for 20 to 30 days.

Protoplast preparation. Sterile protoplasts were prepared by methods modified from Walton and Earle (1985). Leaves (5 g) were abraded with carborundum and incubated at 26°C for 3 h in 2% (w/v) cellulysin in SCM (0.5 M sorbitol, 10 mM $CaCl_2$, and 40 mM Mes), at pH 5.5. Released protoplasts were filtered through 80-μm nylon screen mesh, washed twice with SCM, pH 6.0, and collected by 5 min centrifugation at 100 *g*. In order to measure [^{14}C]Glc incorporation into callose, protoplasts were resuspended in SCM, pH 6.0, at a final concentration of 80,000 protoplasts/ml, and dispensed at 0.5 ml/well into 24-well Falcon multiwell plates (Becton-Dickinson). For treatment of protoplasts for microsome isolation, protoplasts were resuspended in SCM, pH 6.0, at a final concentration of 80,000 protoplasts/ml, and dispensed at 5 ml/ 60 mm petri dish (Falcon).

[^{14}C]Glucose Incorporation Into Callose. Immediately after dispensing protoplasts into the multiwell plates, 0.25 μCi sterile D-[U-^{14}C]Glc in SCM, pH 6.0, was added to each well. The plates were sealed with parafilm and incubated at room temperature in darkness for 2 h. The protoplasts were then transferred to test tubes. The wells were washed twice with 1.0 ml 80 mM citric acid, pH 3.0. The decreased sorbitol concentration caused the

protoplasts to lyse allowing removal of intracellular components.

To remove proteins, 1.0 ml of suspended Dowex 50W cation exchange resin equilibrated with 80 mM citric acid pH, 3.0, was added to each tube containing the protoplasts and two washes. The tubes were rocked for 2 h and the supernatants transferred to 20 ml tubes with one 1.0 ml (80 mM citric acid, pH 3.0) wash of the resin. After addition of ethanol to a final concentration of 70% (v/v, assuming additive volumes), the samples were stored overnight at 4°C, and then collected on Whatman GF/A glass fiber filters using a Millipore model 1225 sampling manifold. The filters were rinsed twice with cold 70% ethanol, and the retained radioactivity was measured using a scintillation counter. The Dowex treatment reduced the background incorporation of untreated protoplasts but not the variability between samples.

Isolation of microsomes. After 2 h incubation of protoplasts in the presence or absence of 6 ng victorin/ml, protoplasts were sedimented by centrifugation at 100 *g* for 5 min. The protoplasts were then kept on ice while resuspending them in 5 ml 50 mM Hepes, 5 mM EDTA, pH 7.5, containing 1 μM leupeptin, 1 μM pepstatin, 1 μM PMSF, 1% polyvinyl pyrrolidone (PVP) 40, and 1 mM DTT. The microsomes were collected by centrifugation in a Beckman Ti 50 rotor for 20 min at 40,000 rpm and 4°C in a Sorvall ultracentrifuge. The microsomal pellet from each plate was resuspended in 0.5 ml 50 mM Hepes pH 7.5, containing 1% PVP, 1 mM DTT, and 1 μM each of leupeptin, pepstatin, and PMSF using a tissue homogenizer. Glycerol was added to a final

concentration of 20%.

Protein Determination. The concentration of protein present in the microsomal preparations was determined by the method of Bradford (1976) using IgG as a standard. Protein concentrations were usually in the range of 1-4 μg/μl.

β (1-3) Glucan synthase assays. Microsomes (100 μl) containing between 100-400 μg protein were incubated in a total volume of 154 μl containing 1.3 mM $CaCl_2$, 6 mM $MgCl_2$, 5.8 mM cellobiose, and 0.026% digitonin. UDP-Glc (192 μM, 0.6 mCi/ mmol) was added to start the reaction (Frost *et al.* 1990). The mixture was incubated at 25°C for 20 min and the reaction was stopped by the addition of 100% ethanol to a final concentration of 66%. A control stopped at 0 min determined background. The reaction mix was filtered through a water-wetted GF/C (Whatman) filter using a Millipore model 1225 sampling manifold. Wetting the filter reduced the binding of unincorporated substrate to the filter thereby reducing background. The filters were rinsed twice with cold 66% ethanol and the retained radioactivity was measured using a scintillation counter.

Chemicals. Digitonin, cellobiose, sorbitol, PVP 40, Dowex 50W cation exchange resin (100-200 mesh, 2% crosslinkage were obtained from Sigma. D-[U-^{14}C]Glc, 258.5 mCi/mmol, and UDP-[^{14}C]Glc, 266 mCi/mmol, were from ICN. $CaCl_2$, $MgCl_2$, and EDTA were purchased from Baker, DTT from Beckman,

Hepes from Research Organics, glycerol from Mallinckrodt, Mes, PMSF, leupeptin, and pepstatin from Boehringer Mannheim. Victorin was purified by the method of Walton and Earle (1984). Red beet plasma membranes and microsomes were the generous gift of Dr. Bruce Wasserman, Rutgers University, NJ.

RESULTS

Maximal callose production by protoplasts occurs following 24 h treatment with 60 pg victorin (Chapter 1; Walton & Earle 1985). To obtain responses to victorin within a shorter time period [^{14}C]Glc incorporation into callose was measured between 0-3 h at several victorin concentrations. Although there was no detectable callose production by protoplasts treated with 60 pg victorin/ml after 3 h (data not shown), protoplasts treated with 6 ng victorin/ml produced 3-6 fold more callose than controls after 2.5 h (Fig. 1).

Microsomal fractions were isolated from protoplasts after incubation in the absence or presence of 6 ng victorin/ml and the amounts of callose synthase activity present in the microsomes of these protoplasts were assayed (Fig. 2A). To determine the amount of callose produced by these cells under identical conditions, protoplasts of the same preparation were incubated with [^{14}C]Glc (Fig. 2B). In six independent experiments, a 4 to 6-fold increase in victorin-induced callose by protoplasts corresponded to a 1.5-fold increase in

Figure 1: Time course for the detectable production of callose by mesophyll oat protoplasts. Protoplasts were incubated in the presence or absence of 6 ng victorin/ml for various times and the amount of radiolabelled callose produced was quantitated.

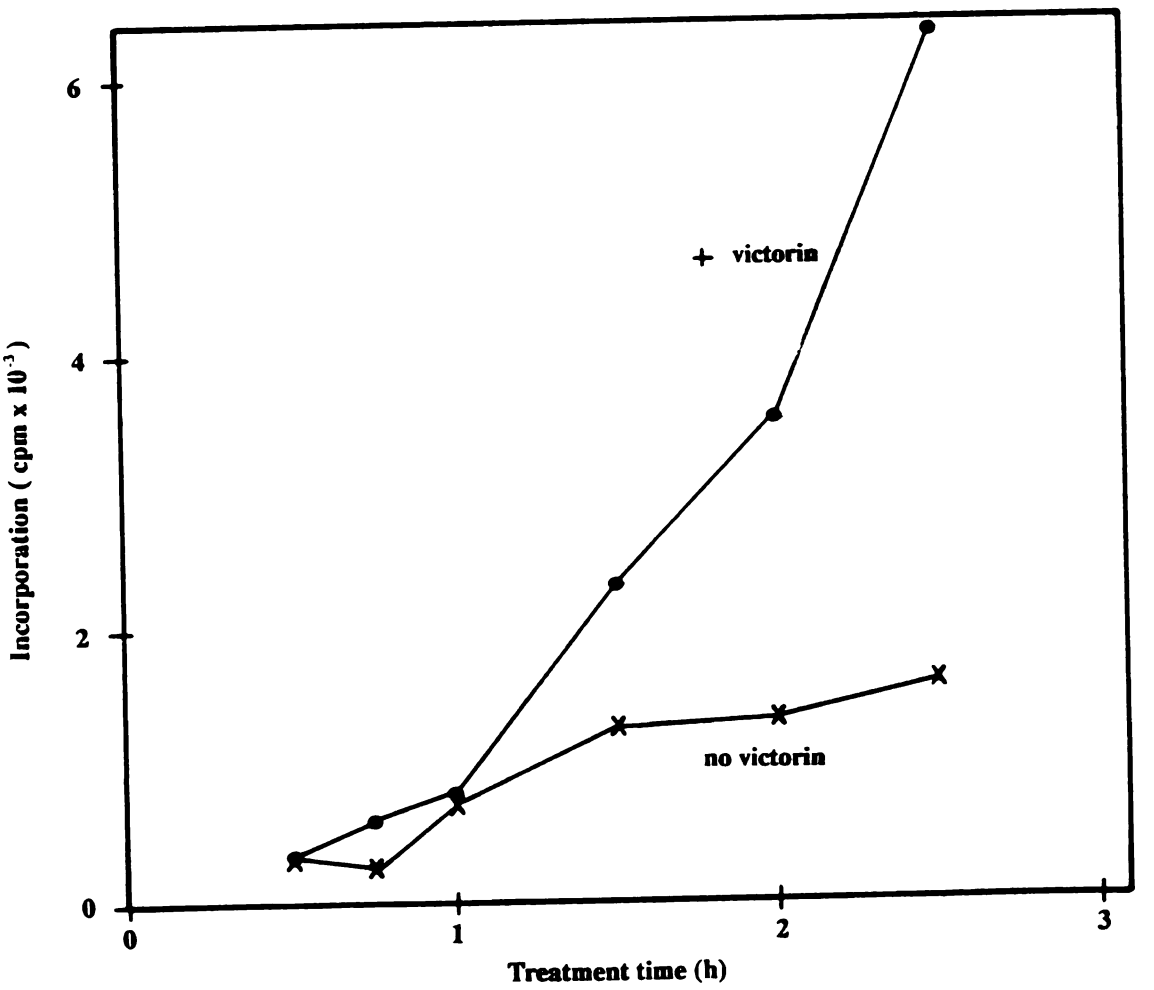
6
4
2
0
Incorporation (cpm x 10^{-3})
+ victorin
no victorin
0
1
2
3
Treatment time (h)

Figure 1

Figure 2: Stimulation of microsomal callose synthase activity in microsomes of victorin-treated protoplasts. A) Microsomes were isolated from protoplasts after 2 h in the absence (C) or presence (V) of 6 ng victorin /ml and assayed for callose synthase activity. The reaction time was varied to determine the linearity of the reaction with time. B) Incorporation of [^{14}C]Glc into callose by protoplasts after 2 h incubation in the absence (C) or presence (V) of 6 ng/ml victorin.

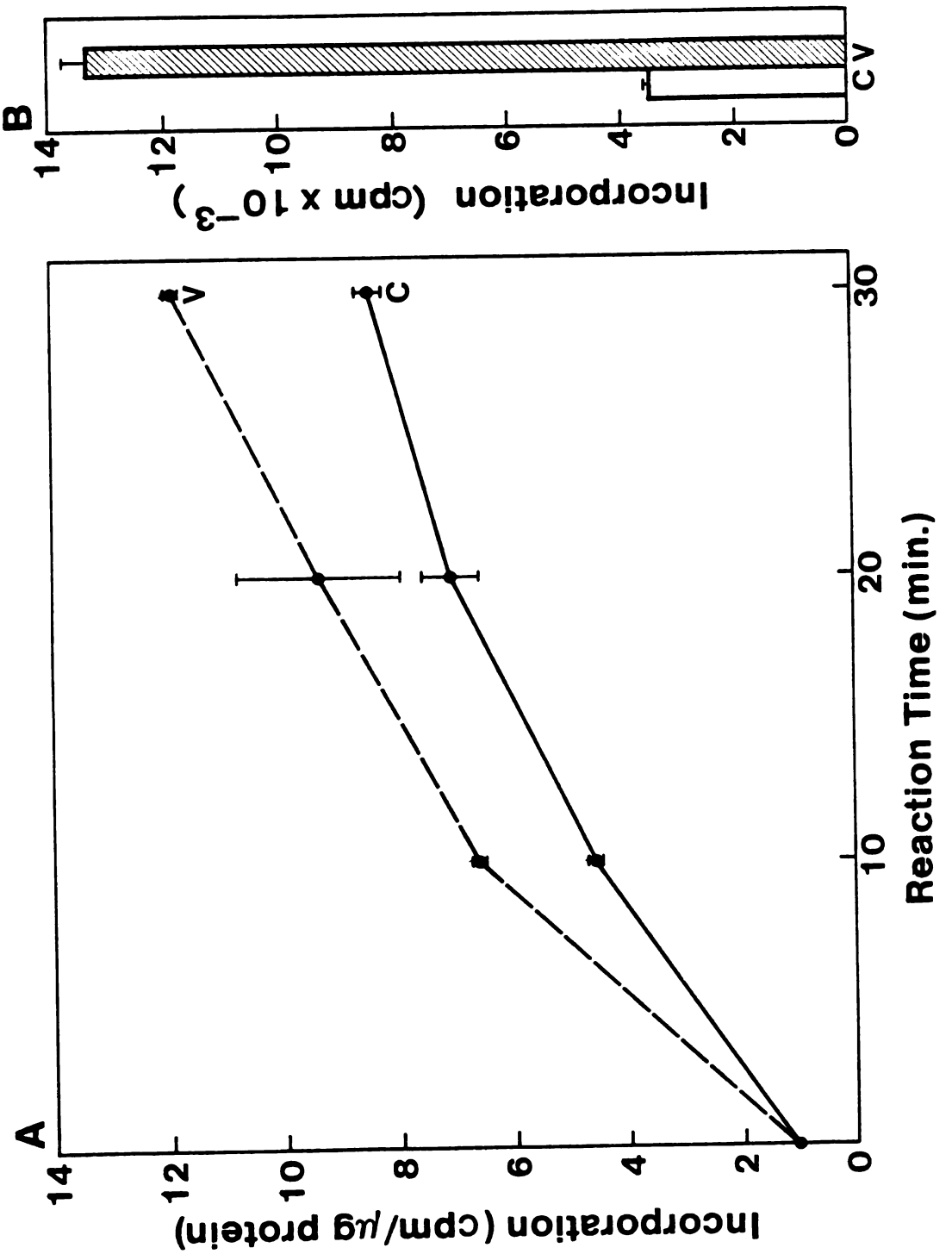
A
Incorporation (cpm/μg protein)
Reaction Time (min.)
V
C
B
Incorporation (cpm x 10^-3)
CV

Figure 2

the detectable microsomal enzyme activity.

The requirements of this microsomal enzyme activity were determinedby assaying the microsomes in the absence of added $CaCl_2$, in the presence of 4 mM EDTA, or in the presence of 6 ng victorin/ml. The results (Fig. 3) show that callose synthase activity is strongly dependent on added Ca^{2+} ions and is inhibited by chelating Ca^{2+} and Mg^{2+} with EDTA. Ca^{2+} has been previously determined to be required for callose synthase activity (Kauss 1987; Delmer 1987a; Fink *et al.* 1990). The addition of victorin to the enzyme assay reduces the callose synthase activity by only 10% to 20%. Therefore, victorin does not act directly on the callose synthase enzyme to increase its activity within the protoplasts since it does not increase the microsomal activity of untreated protoplasts.

The specific activity of callose synthase measured in microsomes of mesophyll tissue is much less than has been observed in other plants (Delmer: cotton and mung bean; Wasserman: red beet; personal communication). To confirm that the low specific activity was not a consequence of the assay conditions, red beet plasma membranes (generous gift of B. P. Wasserman) known to contain a high specific activity of callose synthase were assayed and were determined to contain the same callose synthase activity as that observed by Wasserman (personal communication). In addition, ten to 28-fold more callose synthase activity can be obtained in microsomes of oat root tissue than microsomes from mesophyll protoplasts (Table 1). Victorin also had no effect on the enzyme activity from roots. The microsomes from mesophyll

Figure 3: Influence of Ca^{2+}, EDTA and victorin on microsomal callose synthase activity. Microsomes were isolated from protoplasts after 2 h in the absence (C) or presence (V) of 6 ng victorin /ml. Incorporation of UDP [^{14}C]Glc into callose was measured after 0 min and 20 min. Modifications to the enzyme assay: -Ca, in the absence of added Ca^{2+}; EDTA, in the presence of 4 mM EDTA; HV, in the presence of 6 ng victorin/ml.

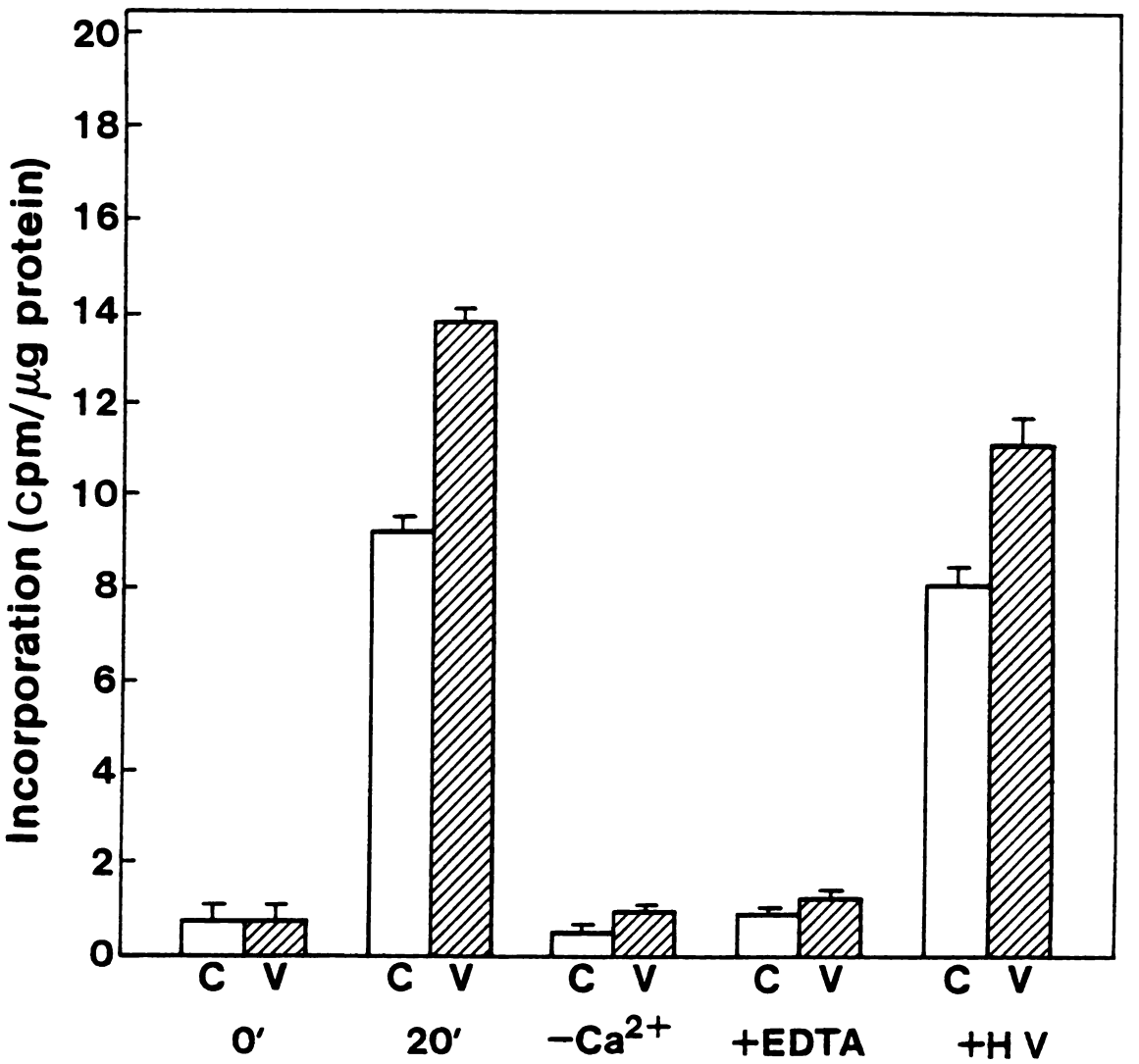
Incorporation (cpm/μg protein)
20
18
16
14
12
10
8
6
4
2
0
C V
C V
C V
C V
C V
0′
20′
−Ca2+
+EDTA
+H V

Figure 3

Table I: Callose synthase activity in microsomes of roots. Microsomes were isolated from roots and mesophyll protoplasts and assayed for callose synthase activity. A mixture of the same amount of root and mesophyll microsomes were assayed to determine if a callose synthase inhibitor was present in mesophyll microsomes. Activity is reported as cpm UDP-Glc incorporation/μg protein. Standard error was less than 5% (n = 2).

microsomes	cpm/μg protein
50μl mesophyll	4
50μl root	106
50μl mesophyll + 50μl root	60

protoplasts do not contain an inhibitor of callose synthase because a mixture of mesophyll and root membranes does not reduce the callose synthase activity of the root microsomes (Table 1).

DISCUSSION

It is not known how callose synthase is regulated within the cell but the various hypotheses suggested in the literature may be tested more easily in a system which is amenable to activity measurements of both the living cells and the isolated membranes. Callose production can be detected within 1.5 h of victorin treatment of mesophyll protoplasts of *Avena sativa* cv Park (Fig. 1). This increased callose production by the protoplasts correlates with an increase in microsomal callose synthase enzyme activity (Fig. 2). This suggests that the callose synthase enzyme responsible for the victorin-induced increased callose biosynthesis can be isolated in the microsomes after victorin treatment of the protoplasts. However, the percentage increase of microsomal activity of the victorin-treated compared to the untreated was less than the measurable callose production by the victorin-treated protoplasts. One difficulty in comparing the microsomal activity with the protoplast produced callose is that callose synthase activity is induced by isolation of microsomes. Although most plant cells do not contain callose, microsomes isolated from them contain callose synthase activity. One possible explanation is that changes to the callose synthase enzyme occur during microsomal isolation. Enzymes present

within the cells may act on the enzyme during isolation, either increasing the amount of enzyme activity present in the microsomes of untreated protoplasts or decreasing the activity present in the microsomes of victorin treated protoplasts. In addition, there may be an induction of callose synthase activity in the untreated protoplasts by the stress to the protoplasts during microsomal isolation. Callose synthase may be regulated within the cell by several mechanisms including post-transcriptional and post-translational control, and association of regulatory molecules or protein subunits which may be disrupted by the isolation procedure. Indirect evidence from purification studies suggests that this enzyme has a complex structure and probably is composed of several subunits with different functions (Delmer 1987a; Fink *et al.* 1990). Therefore, isolation of microsomes may cause removal of regulatory subunits from the complex, changing the ratio of activity between the microsomes of the untreated and treated protoplasts.

Work by Girard and Fevrè (1991b) indicates that a phosphorylated compound is involved in the stimulation of the β (1-4) glucan synthase activity in the Oomycete fungus, *Saprolegnia monoica*. Other research has suggested that other enzymes are also regulated by phosphorylation (Ranjeva & Budet 1987). Since changes in victorin-induced callose synthase in microsomes are not as high as the increase in callose production by the protoplasts, the regulatory mechanism of this enzyme may not be maintained during the microsome isolation procedure. If phosphorylation changes the enzyme from one producing β (1-3)-linked glucan to one producing β (1-4)-linked glucan,

phosphatases would be expected to increase callose synthase activity and this this increased activity would be reversed by kinases. Inhibitors of phosphatases and kinases in the microsomal preparation during isolation would minimize changes during cellular disruption and therefore better maintain the differences observed in the protoplasts.

To test these ideas, microsomes isolated from victorin-treated protoplasts were treated with calf intestine alkaline phosphatase (CIAP; 1U, Boehringer Mannheim) for 30 min at 30°C (by the method of Grab *et al.* 1989) and then assayed for callose synthase activity. CIAP reaction buffer alone inhibited callose synthase activity by 50-60% and CIAP decreased the activity a further 20%. Red beet plasma membranes and microsomes (from B. P. Wasserman) were used to test the effect of CIAP on a more active callose synthase preparation. In two experiments, the callose synthase activity in this system was inhibited by 50-80% by the buffer alone and by 80-100% by buffer and CIAP. However, addition of phosphatase inhibitors $NaMO_4$ and NaF to the phosphatase reaction did not decrease the amplitude of reduction in callose synthase activity. Therefore, the reduction in activity is probably not due to phosphatase activity. Further studies are necessary to understand the factors capable of affecting callose synthase activity in order to more effectively test callose synthase regulation. In addition, because variations in results with this enzyme are evident depending on the plant or plant tissue being used (i.e. Kauss *et al.* 1983 and Sloan & Wasserman 1989), it is important to confirm observations from other work with each plant system analyzed.

Kauss (1987) and others (Delmer 1987; Amor *et al.* 1991) have suggested that callose biosynthesis is based on Ca^{2+} fluxes that may activate or inactivate Ca^{2+}-dependent enzymes. The Ca^{2+} fluxes may not act directly on callose synthase but rather act via a regulatory enzyme. Since some kinases and phosphatases are also regulated by Ca^{2+} ions there may be a complex regulatory system involved in callose induction in plants. Recent work by Delmer and colleagues (1991) suggests the complexity. From proteins eluted from cotton membranes there is 1) kinase activity, 2) a GTP-binding protein with possible similarity to alpha subunits of G-proteins, 3) a 34 kD peptide containing "striking homology" to a conserved region of protein kinase C and annexins, and 4) addition of these proteins enriched for the 34 kD peptide stimulates callose synthase activity under conditions where $Ca^{2+}$is limiting. Although all of these observations may not be directly connected with callose biosynthesis, the complexity of callose synthesis and its regulation are evident.

To date, there are few data to support or refute the hypothesis that callose and cellulose synthase are the same enzyme. The initial expectation was that if callose and cellulose are produced by the same enzyme, then there would be no difference in the callose synthase activity of untreated and victorin-treated protoplasts. However, if the enzymes are different, then the activity would be equal to the increase in callose observed in the victorin-treated protoplasts. The results described herein do not permit a clear distinction but rather suggest a more complex regulation of callose biosynthesis during biotic and abiotic stresses.