140 and 220 bp smaller, respectively, than B95-8 and must therefore contain a smaller IR3. Since the B95-8 Msp I fragment is larger because of a longer IR3, the Msp I fragment of Cherry is likely to be largest in that it contains the longest IR3.

The size of the EBNA protein in cell lines infected with each of the EBV isolates was determined by incubating EBNA positive human serums with immunoblots of cell protein extracts (Fig. 2, B to D). The size of B95-8 and IB4 EBNA are 75K; near the size previously determined for B95-8 with markers of different sizes (*15*). On the basis of the difference in size among Msp I DNA fragments, the size of Raji, Lamont, and Cherry EBNA proteins would be expected to be 69K, 71K, and 83K, respectively. These values agree well with the 68K, 72K, and 85K of these proteins relative to protein standards in denaturing polyacrylamide gels (Fig. 2B). Thus, the range of IR3 and EBNA polymorphism is at least 500 bp and 17K, respectively. Five serums from EBNA-positive (ACIF) patients react with the polymorphic EBNA protein. Five EBV immune, EBNA ACIF negative human serums do not react with this protein. Neither type of serum reacts with proteins of the EBV-negative B cell line Louckes. The EBNA protein is greatly enriched in the nuclear fraction (Fig. 2D; data not shown).

These data indicate that (i) the 2-kb exon of the 3.7-kb latently infected cell RNA maps entirely within the 2.8-kb Bam HI–Hind III fragment, which contains IR3 and (ii) IR3 encodes part of EBNA. Variation in size of IR3 provides the mechanism by which EBNA polymorphism is generated. Since IR3 is an entirely open reading frame and is transcribed from left to right, the peptide encoded by IR3 first, second, or third reading frames, respectively, would consist of serine, arginine, and glycine; glycine and alanine; or glutamine, glutamate, and glycine. There are translational termination codons within 250 bp to the left and right of IR3 in the first and third reading frames (*11*). There is one possible, but unlikely, splice site that could eliminate stop codons (*11, 19*). If this unlikely candidate splice acceptor site is not functional, the first and third reading frames are excluded since the size of EBNA is approximately 80K and EBNA is encoded in part by IR3. The second reading frame encodes a glycine alanine copolymer which would then represent approximately 19K of the 75K B95-8 EBNA protein. Further evidence that this region encodes EBNA is that transfection of the Bam HI K fragment or of its leftward Bam HI–Hind III subfragment, which contains IR3 (Fig. 1), into L cells has been reported to induce an intranuclear antigen detectable only with EBNA-positive human serums (*20*).

Our result indicates that EBNA has a core of simple amino acid composition. Because of its binding to chromosomes, EBNA may participate in the regulation of cell growth (*15, 16*). The identification of a copolymer domain in EBNA is a step in defining the protein component of this interaction.

Uninfected human and mouse cell DNA's contain an interspersed repeat sequence with a high degree of homology to EBV IR3 (*21*). Although no IR3 homologous RNA has been detected in the non-EBV infected Louckes B cell line (*11*), it remains to be seen whether cellular IR3 homologs encode an EBNA-related protein at some stage of development.

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Sucrose: A Constitutive Elicitor of Phytoalexin Synthesis

Abstract. *Extracts of seeds and leaves of the tropical legume* Cajanus cajan *(L.) Millsp. (the pigeon pea) elicited the accumulation of three phytoalexins when applied as droplets to superficially wounded leaves of the plant. The active component was purified and identified as sucrose. Phytoalexin accumulation was proportional to the logarithm of the concentration of sucrose applied, with maxima ranging from 338 to 455 micrograms per gram (fresh weight) of leaf tissue. The sucrose concentrations required to elicit half these amounts ranged from 20 to 35 micrograms per milliliter, but other sugars had little effect even at 1000 micrograms per milliliter. The elicitor activity of sucrose was abolished by actinomycin D, puromycin, and cycloheximide at a concentration of 10 micrograms per milliliter or greater, suggesting that gene derepression is required for expression of the phytoalexin response.*

Phytoalexins are antimicrobial compounds of low molecular weight that are synthesized by and accumulate in plants after exposure to microorganisms (*1*). The role of these compounds as antiparasitic agents in some plants is supported by considerable evidence (*2, 3*), but little is known about the mechanisms that control their production. They may be synthesized specifically in response to avirulent races of pathogenic fungi (*4*) but not to virulent races of the same organism (*4, 5*), and yet they also accumulate as a result of treatment with such nonspecific agents as the salts of heavy metals (*6*), ultraviolet radiation (*7*), and chloroform vapor (*8*).

A solution to this paradox was provided by Bailey (*9*), who showed that plants contain a constitutive compound that may function as a phytoalexin elicitor (*10*). It is thought that this compound diffuses from damaged cells and elicits phytoalexin synthesis in adjacent healthy cells. Thus damage, whether caused by avirulent races of pathogens or by less subtle means, may trigger phytoalexin synthesis. Recent work (*11*–

13) indicated that in soybean and castor bean plants, cell wall fragments can function as elicitors but that their release by enzymatic action is obligatory for activity. In contrast, we now report a constitutive phytoalexin elicitor that is present in the free form in the intact plant.

Recently we found that leaves of the pigeon pea [*Cajanus cajan* (L.) Millsp.] synthesize three phytoalexins in response to inoculation with the fungus *Botrytis cinerea*, which is avirulent for this plant. One was the known compound pinostrobin chalcone, but the other two were novel isomeric stilbene-2-carboxylic acids (*14*). All three compounds also accumulated in response to extracts of both leaves and imbibed seeds of the plant.

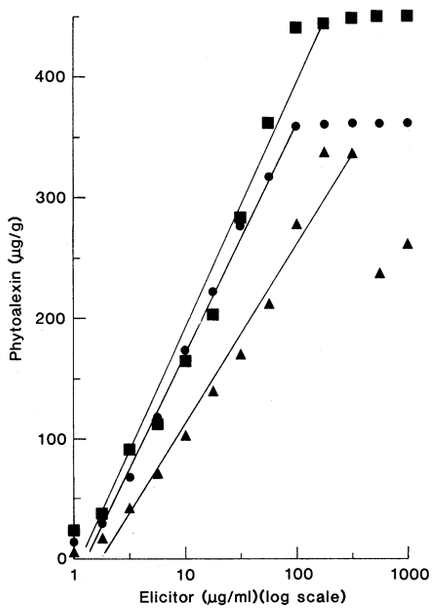

Fig. 1. Accumulation of phytoalexins by pigeon pea leaves in response to purified constitutive elicitor from seeds. Leaves were surface-sterilized in hydrogen peroxide (25 volumes) for 30 minutes, rinsed in three changes of sterile distilled water, and wiped dry with sterile muslin. The adaxial epidermis was abraded with single strokes of the tip of a sterile syringe needle, giving 20 wounds 2 to 3 mm in length per leaf. The wounds were located midway between the lateral veins and ran parallel to them. Droplets (20 μl) of elicitor preparation (sterilized by filtration) or sterile distilled water were placed on each wound, and the leaves were incubated at high humidity for 72 hours in the dark at 25°C. Leaves were extracted in 95 percent ethanol by the facilitated diffusion technique (*15*), and the phytoalexins were assayed by analytical high-pressure liquid chromatography on a Hypersil ODS column (length, 250 mm; inner diameter, 4.6 mm) with acetonitrile, water, and acetic acid (65:35:1, by volume) as the solvent. Each point is the mean of three replicates. Standard deviations never exceeded 18.3 μg of phytoalexin per gram (fresh weight) of leaf tissue. Symbols: (●) compound 1 (pinostrobin chalcone), (▲) compound 2, and (■) compound 3 (see Fig. 3 for molecular structures).

Seed extracts (95 percent ethanol) were purified by thin-layer chromatography [Si-gel with *n*-butanol, diethyl ether, acetic acid, and water (90:60:30:10, by volume) as the developing solvent], and the active area on the plate was eluted in methanol. After evaporation of the methanol, the residue was taken up in water and filter-sterilized. Phytoalexin elicitation was proportional to the logarithm of the concentration of this preparation (Fig. 1). Maximum yields after incubation for 72 hours ranged from 338 to 445 μg of phytoalexin per gram (fresh weight) of leaf tissue, depending on the compound, and the doses of the elicitor preparation required to give half these maxima (the ED_{50} values) were 22.5 μg/ml for compound 1 (pinostrobin chalcone), 43.8 μg/ml for compound 2, and 32.5 μg/ml for compound 3 (Fig. 1).

The ^1H nuclear magnetic resonance (NMR) spectrum of the elicitor fraction suggested that it was composed of carbohydrates and that it consisted largely of one compound, a finding borne out by the ^{13}C NMR spectra. Comparison of the ^{13}C and ^{13}C INEPT (*16*) NMR spectra with those of sucrose indicated that sucrose was the major component. This identification was substantiated by a comparison of the ^1H NMR spectrum of the elicitor compound and sucrose and by the retention time in gas-liquid chromatography of octatrimethylsilylsucrose and a trimethylsilylated sample of the major component.

When sucrose was applied to wounded sterile leaves in the assay, phytoalexins became detectable within 24 hours and reached maximum values after 72 hours (Fig. 2). Phytoalexin yields were proportional to the logarithm of the concentration of sucrose, and the ED_{50} values were 30.3, 34.8, and 20.5 μg/ml for compounds 1, 2, and 3, respectively, when measured after incubation for 60 hours. Of a number of neutral sugars tested (glucose, fructose, galactose, maltose, raffinose, stachyose, and turanose), only sucrose had significant elicitor activity.

Incorporation of the inhibitors actinomycin D, cycloheximide, and puromycin with sucrose in the assay inhibited phytoalexin accumulation, although at low concentrations all three compounds alone elicited some phytoalexin synthesis (Fig. 3). In contrast, the addition of nystatin (5730 U/ml) or streptomycin (735 U/ml) had no effect on the elicitor activity of sucrose.

Our finding that sucrose is a constitutive elicitor of phytoalexin synthesis may explain a number of previous observations. For example, Robinson and Wood (*17*) observed that leaf disks of the pea plant (*Pisum sativum* L.), when floated on sucrose solutions, accumulated the pterocarpan phytoalexin pisatin, and Russell (*18*) and Cartwright and Russell (*19*) reported that the resistance of plants to fungal parasites was increased by spraying with sucrose before inoculation.

Many plants contain high concentrations of constitutive antimicrobial compounds, presumably to ward off attacks by microorganisms (*20*). Phytoalexin accumulation appears to be a more economical means of achieving the same end, since the compounds are synthesized close to the point of attack, where they remain (*9*). The problem for the plant, therefore, is to recognize and respond swiftly to the attacking microorganism by producing inhibitory concentrations of phytoalexin. Our results suggest that, at least in the pigeon pea, the plant is sensitive to the dislocation of sucrose. Sucrose is generally abundant in plants and may be found in high concentrations in the phloem as well as in the apoplast and vacuoles (*21*). Estimation of sucrose concentrations in leaves and seeds of the pigeon pea by the method of Hümme *et al.* (*22*) gave values of 1.04 ± 0.33 and 7.47 ± 2.45 mg/g (fresh weight), respectively. As shown in this study, diffusion of only a few micro-

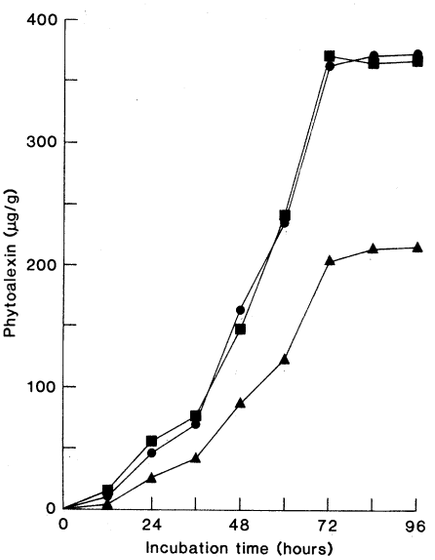

Fig. 2. Relation of phytoalexin accumulation to duration of incubation with sucrose (100 μg/ml). The experimental procedures are described and the symbols are identified in the legend to Fig. 1. Standard deviations never exceeded 6.4 μg of phytoalexin per gram (fresh weight) of leaf tissue.

grams of sucrose is sufficient to elicit considerable phytoalexin synthesis, implying the existence of a receptor not normally accessible to sucrose in the intact plant. We suggest that binding of sucrose to this receptor in some way derepresses phytoalexin synthesis. Considering the relative concentrations of sucrose in the plant and the amount required to elicit phytoalexin synthesis, the mechanism would appear to be delicately poised. Low concentrations of actinomycin D, cycloheximide, and puromycin, for example, appear to be sufficient to trigger phytoalexin production, although higher concentrations of these compounds depress the elicitor activity of sucrose, presumably because of their effects on RNA and protein synthesis. How successful parasites avoid activating the response is an intriguing question; it is perhaps significant that some are known to secrete invertases (*23, 24*).

With the establishment of sucrose as a constitutive elicitor of phytoalexins in the pigeon pea, a broad investigation of phytoalexin production can be envisaged. The range of plants in which sucrose is an effective elicitor is of interest, and preliminary experiments have shown that four other leguminous plants—peanut, soybean, mung bean, and French bean—accumulate antifungal compounds when droplets of sucrose solution are applied to wounded leaves. Similarly, antifungal compounds accumulate in pea leaves treated in the same manner, confirming the results of Robinson and Wood (*17*).

Of more fundamental importance will be the determination of the location and nature of the sucrose receptor. One hypothesis is that the receptor is a repressor molecule which, if combined with sucrose, allows transcription of the genes specifying the enzymes necessary for phytoalexin synthesis. As already stated by Grisebach and Ebel (*25*), phytoalexin biosynthesis may provide great possibilities for studying the control of gene expression in plants, particularly since the phytoalexin response may be triggered by such a nontoxic compound as sucrose.

Finally, our observations may be helpful to plant breeders seeking to incorporate disease resistance into crop plants. Where these contain sucrose as the constitutive phytoalexin elicitor, breeders could select for plants with high sucrose levels and a well-developed capacity to respond to sucrose dislocation by the rapid accumulation of antimicrobial concentrations of phytoalexins.

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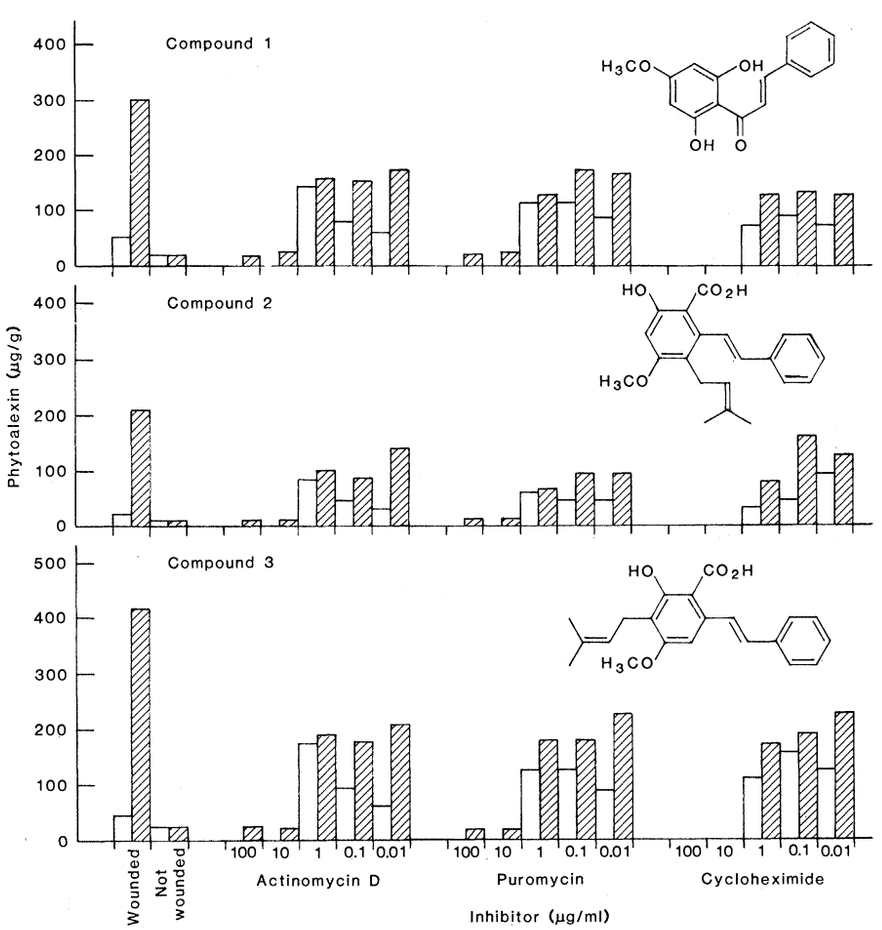

Fig. 3. Effect of actinomycin D, puromycin, and cycloheximide on the elicitation of phytoalexins by sucrose. Inhibitors were tested separately (open bars) and mixed with sucrose (100 μg/ml) (shaded bars) in accordance with the procedure described in the legend to Fig. 1. The incubation time was 72 hours. Column heights represent the mean of three replicates. Standard deviations never exceeded 7.0 μg of phytoalexin per gram (fresh weight) of leaf tissue.

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