Selective Degradation of Proteins by *Cercospora kikuchii* and *Phomopsis longicolla* in Soybean Seed Coats and Cotyledons

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ABSTRACT

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Soybean seeds infected with *Cercospora kikuchii* showed degradation of seed coat proteins but not cotyledonary proteins, whereas seeds infected with *Phomopsis longicolla* showed degradation of seed coat and cotyledonary proteins. Lipoxygenase, conglycinins (α, α', β, β'), and glycinins (A_1, A_4), which are cotyledonary proteins degraded by *P. longicolla*, were tentatively identified by their molecular weights. Lipoxygenase was degraded only in seed coats infected with *C. kikuchii* and in cotyledons infected with *P. longicolla*. β-Amylase was partially degraded in seed coats and cotyledons infected with *P. longicolla* but not in seeds infected with *C. kikuchii*. Soybean seed lectins were found in seed coats infected with *P. longicolla* but not in seeds infected with *C. kikuchii* or in the uninfected control. Soybean seed lectins were not degraded in cotyledons of seeds infected with either fungus.

Cercospora kikuchii (Matsumoto & Tomoyasu) M. W. Gardner causes purple seed stain of soybeans (*Glycine max* (L.) Merr.) and enters the seed coat through the funiculus (18). The pathogen grows intercellularly in plant tissues, wherein nutrients are acquired through membrane leakage caused by the toxin cercosporin (19). *Phomopsis longicolla* T. W. Hobbs infects soybean seeds during or after the yellow pod stage (R7) and causes seed decay (18). The pathogen infects all parts of the soybean seed, including the seed coat, cotyledons, and embryo. Severely infected seeds are shriveled, elongated, and appear white or chalky. Infected seeds germinate poorly, and the seedlings can be destroyed by pre- or postemergence damping-off.

Soybean seeds contain a variety of proteins, including storage globulins, lectin, lipoxygenase, β-amylase, etc. (3). Storage globulins consist of two major classes of proteins, glycinins (11S) and conglycinins (7S). β-Conglycinins constitute 30% of the total protein and are composed of four subunits, denoted α, α', β, and β'. Glycinins include several acidic and basic subunits (13). Structural and quantitative variations in these two protein groups influence nitrogen and sulfur content and functional properties of soybean foods, such as solubility, coagulation temperature, and flavor.

The lipoxygenases are monomeric globular proteins with molecular weight of approximately 100 kDa (2), are present in soybean cotyledons (1), and constitute 1–2% of the total seed protein (22). Lipoxygenases may contribute to resistance against pathogens (11,14,15)

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or function as storage proteins (16).

Soybean seed lectin (SBL) is a 120-kDa tetrameric glycoprotein with 30-kDa units or 15-kDa subunits and is principally found in the cotyledons (5,7,20). Higher levels of SBL in seeds of cultivars resistant to *Phytopthora sojae* M. J. Kaufmann & J. W. Gerdemann than in seeds of susceptible cultivars suggested a possible role of lectins in disease resistance (6). β-Amylase is a sulfhydryl-containing enzyme with a molecular weight of approximately 60 kDa (12); it functions in starch degradation.

We report on the association of selective degradation of certain proteins by *C. kikuchii* and *P. longicolla* in soybean seed coats and cotyledons. The effects of this degradation on seed germination, seed quality, and seedling mortality are discussed.

MATERIALS AND METHODS

Detection and isolation of *C. kikuchii* and *P. longicolla*. Soybean seeds with and without characteristic symptoms induced by *C. kikuchii* or *P. longicolla* were selected from seed lots of cv. Hack (Illinois Seed Foundation, Tolono, IL.). The seed lots had been stored in the dark at 4 C for 1 yr. Asymptomatic and symptomatic seeds were surface-disinfected separately in 0.5% NaOCl for 5 min and placed on Difco potato-dextrose agar (pH 7) (PDA) (Difco Laboratories, Detroit, MI) in 9-cm-diameter plastic culture plates, with three seeds per plate in 50 replications for purple-stained seeds and nine replications for seeds infected with *P. longicolla*. The plates were incubated under continuous fluorescent light (160 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 30 days at room temperature (24 ± 2 C). Percentages of seed germination and seedling mortality were recorded after 5 and 10 days, respectively. The two fungi were identified after 7 days and were subcultured.

Proteins from fungi, asymptomatic and symptomatic soybean seed coats, and cotyledons. *C. kikuchii* and *P. longicolla* were subcultured on culture plates containing 20 ml of PDA and incubated as described previously. Mycelium scraped from the PDA surface was dried in the dark for 48 hr at 24 ± 2 C and used as a control to simulate natural drying of the fungi in seeds. The dried mycelium was ground in liquid N_2 and weighed. Asymptomatic and symptomatic seeds infected with *C. kikuchii* or *P. longicolla* were cracked in a mortar, and seed coats were separated from the cotyledons. Five seeds were used for each experiment, and three independent experiments were done.

The funiculus of the seed coats and the axis of the embryos were removed and discarded. The seed coat and cotyledonary tissue were ground separately in liquid N_2 and stored at 4 C. The ground tissue (20 mg/ml) samples were mixed in a buffer (62.76 mM Tris-HCl [pH 6.8], 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, and 10% glycerol), held in boiling water for 5 min, and then centrifuged at 10,000 *g* for 2 min. We conducted SDS-polyacrylamide gel electrophoresis (PAGE) on discontinuous polyacrylamide slab gels by using a Bio-Rad PROTEAN II xi slab cell (Bio-Rad Laboratories, Richmond, CA) (10). Ten microliters containing 20 μg of cotyledon extract, 50 μl containing 100 μg of seed coat extract, or 30 μl containing 60 μg of mycelial extract from each fungus was loaded onto 12–17% gradient gels, and electrophoresis was conducted at a constant voltage (200 V). Gels were stained with Coomassie brilliant blue R-250 and scanned on an LKB UltroScan XL laser densitometer (LKB, Bromma, Sweden). Protein bands were identified according to Tae-Jucho et al (21).

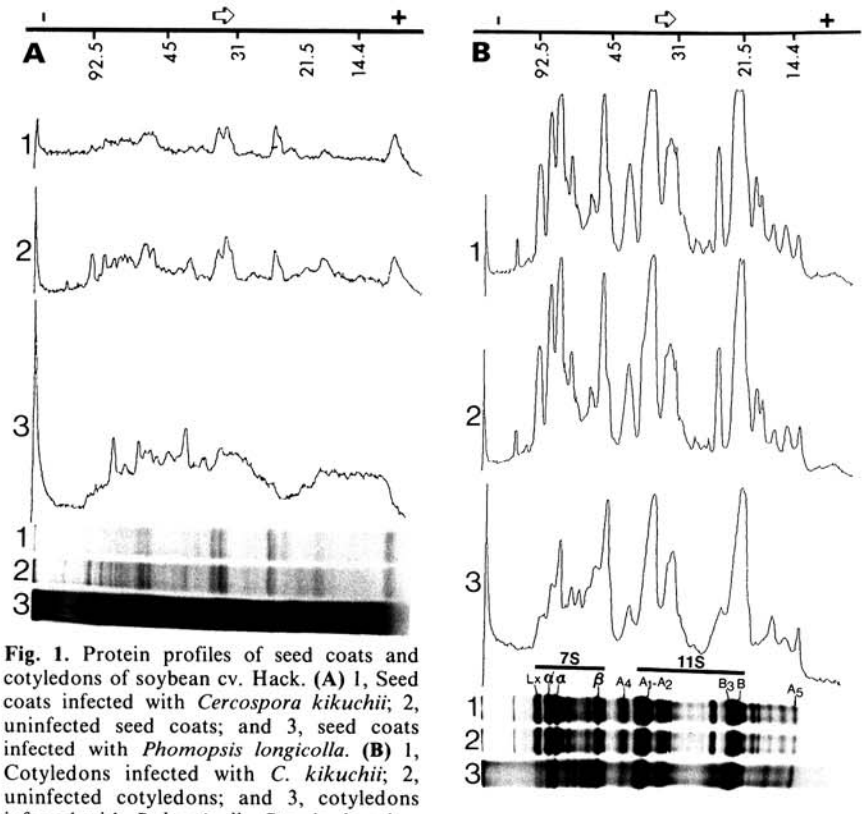

Fig. 1. Protein profiles of seed coats and cotyledons of soybean cv. Hack. **(A)** 1, Seed coats infected with *Cercospora kikuchii*; 2, uninfected seed coats; and 3, seed coats infected with *Phomopsis longicolla*. **(B)** 1, Cotyledons infected with *C. kikuchii*; 2, uninfected cotyledons; and 3, cotyledons infected with *P. longicolla*. Standard molecular weights are given above (in kilodaltons).

Immunoblot analysis of lipoxygenase and soybean seed lectins. The mycelia of *C. kikuchii* or *P. longicolla* and the soybean seed coats or cotyledons of asymptomatic or symptomatic seeds were ground separately in liquid N_2 and extracted with buffer as described previously. Five seeds were extracted for each experiment, and two independent experiments were conducted. Ten-microliter samples containing either 2 mg/ml of cotyledon extract, 20 mg/ml of seed coat extract, or 20 mg/ml of mycelial extract were applied per lane and resolved by 12% PAGE with a Bio-Rad mini-PROTEAN II Dual slab cell. After electrophoresis, we transferred the fractionated proteins to a nitrocellulose membrane by using a transfer buffer (0.048 M Tris, 0.039 M glycine, 0.0375% [w/v] SDS, and 20% methanol [pH 9.0]). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.02 M Tris and 0.18 M NaCl for 3 hr followed by an 8-hr incubation with 0.5 μl/ml of rabbit anti-lipoxygenase polyclonal antibodies in TBS with 0.1% Tween 20 (TTBS). The membranes were washed four times in TTBS for 15 min each time, incubated 3 hr in goat anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase at a dilution of 1:1,000 in TTBS, and rinsed four times in TTBS for 15 min each time. The membranes were developed by using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (17). The quantities of each sample and the procedure used for immunostaining of SBL with rabbit anti-SBL antibodies were the same as those used for lipoxygenases.

Detection of β-amylase activity. β-Amylase activity in asymptomatic and symptomatic soybean seed coats and cotyledons infected with *C. kikuchii* or *P. longicolla* or their respective mycelia was determined by a gel-staining technique (8). Samples of either fungal or seed material (20 mg) were incubated overnight in 1 ml of a buffer (0.092 M Tris [pH 8.1], 0.023 M $CaCl_2$, and 0.5

mM phenylmethylsulfonyl fluoride) at 4 C, and then centrifuged at 10,000 *g* for 5 min. The supernatant was mixed with an equal volume of sample buffer (20% glycerol and 0.01% bromophenol blue) before electrophoresis. A 10-μl sample of extracts from seed coats or fresh mycelium, 4 μl of cotyledon extracts, or 5 μl of an extract from cv. William 82 cotyledons (positive control) was loaded separately in each lane of a 10% nondenaturing polyacrylamide gel. Using a Bio-Rad mini-PROTEAN II Dual slab cell, we conducted electrophoresis under nondenaturing conditions (4). After electrophoresis, the gels were incubated in 0.75% soluble starch in 50 mM sodium acetate (pH 5.0) for 20–30 min at room temperature. The gels were then briefly rinsed with deionized distilled water and stained with 0.1% iodine and 0.5% potassium iodide in 0.5% glacial acetic acid. The β-amylase activity was visualized as clear bands on a blue background.

RESULTS

***C. kikuchii* and *P. longicolla* from symptomatic seeds.** *C. kikuchii* grew from all the purple-stained seeds, and *P. longicolla* grew from whitish, chalky, and shriveled seeds. *C. kikuchii* was not recovered from asymptomatic seeds. However, *P. longicolla* was recovered from 10% of the asymptomatic seeds. All the seeds infected with *C. kikuchii* germinated, similar to asymptomatic seeds, whereas only 40% of the seeds infected with *P. longicolla* germinated. Seedlings from seeds infected with *P. longicolla* were weak and eventually died.

Proteins from fungi, seed coat, and cotyledons of asymptomatic and symptomatic seeds. Degradation of proteins in seed coat samples was detected in seeds infected with *C. kikuchii* (Fig. 1A). The protein profiles of seed coats infected with *P. longicolla* appeared as a smear, suggesting severe degradation of proteins. Degradation of cotyledonary proteins was not observed in seeds infected with *C. kikuchii*. The cotyledons of seeds infected with *P. longicolla* showed selective degradation of proteins at MWs of 91.5, 81.5, 77, 73, 70, 56.5, 62, 22, 19, and 16 kDa; in addition, a general protein degradation is shown by a densitometric scan (Fig 1B). Some of the storage globulins degraded by *P. longicolla* were tentatively identified as conglycinins (α, α', β, β') and glycinins (A_1, A_4).

Lipoxygenase in asymptomatic and symptomatic seed tissues. Extracts from cotyledons infected with *C. kikuchii* showed a slight increase in lipoxygenase as compared to uninfected controls (Fig. 2). However, extracts from cotyledons infected with *P. longicolla* showed degradation of lipoxygenases. Extracts from seed coats infected with *C. kikuchii* also showed degradation of lipoxygenases as compared to uninfected controls. A slight increase in lipoxygenase was observed in seed coats infected with *P. longicolla*. Lipoxygenases were not present in the mycelia of either fungus.

SBL in asymptomatic and symptomatic seed tissues. The 30-kDa SBL subunit observed in extracts of cotyledonary tissue infected with *C. kikuchii* was similar to that observed in seeds infected with *P. longicolla* and in the uninfected controls (Fig. 3). SBL was present in seed coats infected with *P. longicolla* but not in seed coats infected with *C. kikuchii* or uninfected seeds. SBL was not detected in the mycelia of either fungus.

β-Amylase activity of asymptomatic and symptomatic seed tissues. β-Amylase activity was observed in the extracts of seed coats of uninfected seeds and those from seeds infected with *P. longicolla*, but not from seed coats infected with *C. kikuchii* (Fig. 4). Extracts of cotyledonary tissue of seeds infected with *C. kikuchii* showed no difference in β-amylase activity when compared to uninfected positive controls

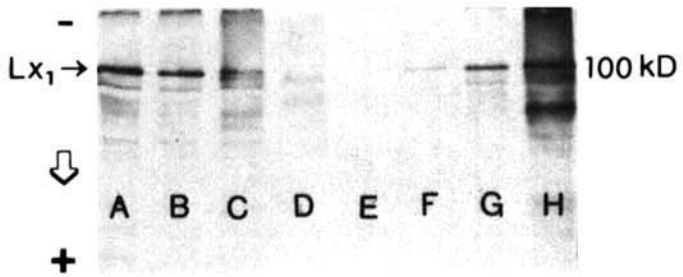

Fig. 2. Immunoblot analysis of lipoxygenase (Lx_1) in seed coats and cotyledons of soybean cv. Hack. **(A)** Cotyledons infected with *Cercospora kikuchii*; **(B)** uninfected cotyledons; **(C)** cotyledons infected with *Phomopsis longicolla*; **(D)** mycelia of *P. longicolla*; **(E)** mycelia of *C. kikuchii*; **(F)** seed coats infected with *C. kikuchii*; **(G)** seed coats of uninfected seeds; and **(H)** seed coats infected with *P. longicolla*. Molecular weight of lipoxygenase indicated on right.

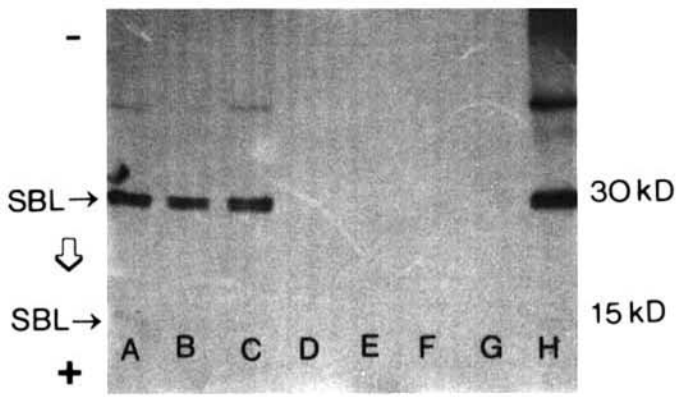

Fig. 3. Immunoblot analysis of soybean seed lectins (SBL) in seed coats and cotyledons of soybean cv. Hack. **(A)** Cotyledons infected with *Cercospora kikuchii*; **(B)** uninfected cotyledons; **(C)** cotyledons infected with *Phomopsis longicolla*; **(D)** mycelia of *P. longicolla*; **(E)** mycelia of *C. kikuchii*; **(F)** seed coats infected with *C. kikuchii*; **(G)** uninfected seed coats; and **(H)** seed coats infected with *P. longicolla*. Molecular weights of SBL monomer and subunits are indicated on the right.

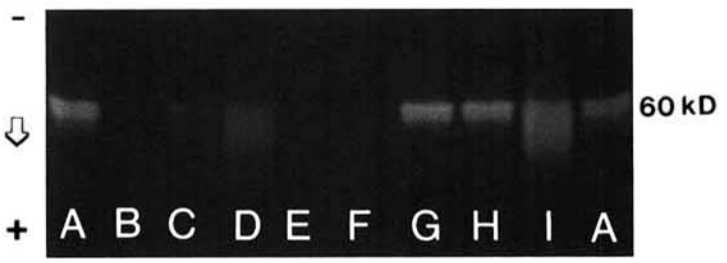

Fig. 4. Analysis of β-amylase activity in seed coats and cotyledons of soybean cv. William 82: **(A)** uninfected cotyledons (positive control); and cv. Hack: **(B)** seed coats infected with *Cercospora kikuchii*, **(C)** uninfected seed coats, **(D)** seed coats infected with *Phomopsis longicolla*, **(E)** mycelia of *P. longicolla*, **(F)** mycelia of *C. kikuchii*, **(G)** cotyledons infected with *C. kikuchii*, **(H)** uninfected cotyledons, and **(I)** cotyledons infected with *P. longicolla*. Molecular weight of β-amylase indicated on the right.

of cvs. Hack and Williams 82. β-Amylase activity in seed coat and cotyledonary tissue extracts of seeds infected with *P. longicolla* appeared as a smear, which is probably due to a partial structural degradation. β-Amylase activity was not observed in either fungus grown on PDA.

DISCUSSION

The degradation of structural and functional proteins in soybean seed parts infected with *C. kikuchii* and *P. longicolla* affected both seed quality and viability. Increased quantities of lipoxygenases and SBL in the seed coats infected with *P. longicolla* suggested a possible role in pathogenesis.

The germination and survival of soybean seeds infected with *C. kikuchii* kept under continuous light for more than 30 days suggested that *C. kikuchii* or its toxin, cercosporin, had an insignificant effect on soybean seed germination or seedling mortality. However, the poor germination of seeds infected with *P. longicolla* may be explained, in part, by the phytotoxic effect of the metabolites produced by this pathogen in culture (9).

Degradation of proteins in seed coats infected with *C. kikuchii* could be due to cercosporin, because this photoreactive toxin oxidizes cell and organelle membranes (19). Histopathological studies of soybean seed coats infected with *C. kikuchii* compared with uninfected controls showed no distortion in the seed coat anatomy (*unpublished*). Reduced germination and/or development of weak seedlings in seeds infected with *P. longicolla* could be a result of the severe degradation of seed proteins.

The qualitative and quantitative changes in glycinin and conglycinin storage proteins of seeds infected with *P. longicolla* can reduce the quality of soybean foods. Quantitative variations in glycinin, which contains 2–3 times more cystine and methionine per unit of protein than conglycinin, affect textural characteristics, gelling time, sulfur and nitrogen content, hardness of tofu gels, and curd formation (13). The increase in lipoxygenase in seed coats of seeds infected with *P. longicolla* was similar to the induction of lipoxygenase activity in tobacco leaves after infection with *Erysiphe cichoracearum* (11). The degradation of lipoxygenase in seed coats of seeds infected with *C. kikuchii* could be a result of cercosporin activity.

The presence of SBL only in soybean seed coats infected with *P. longicolla* suggested that SBL may have been induced by the pathogen or may have leaked from the cotyledons during pathogenesis. Degradation of SBL was not recorded in cotyledons infected with *P. longicolla*. High levels of SBL in soybean seeds of cultivars resistant to *P. sojae*, which inhibited mycelial growth, indicated a possible role of SBL in disease resistance (6). However, the physiological or pathological role of SBL in soybean seeds is not clear. The significance of reduced β-amylase activity in seed coats of seeds infected with *C. kikuchii* and cotyledons of seeds infected with *P. longicolla* is also not clear.

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