MPMI Vol. 8, No. 3, 1995, pp. 388–397.

The *Fusarium solani* Gene Encoding Kievitone Hydratase, a Secreted Enzyme that Catalyzes Detoxification of a Bean Phytoalexin

Daoxin Li, Kuang-Ren Chung, David A. Smith and Christopher L. Schardl

Department of Plant Pathology, S-305 Ag. Science Bldg.-N, University of Kentucky, Lexington, KY 40546-0091 U.S.A.

Received 11 July 1994. Accepted 7 February 1995.

Among the antimicrobial phytoalexins produced by *Phaseolus vulgaris* (French bean) is the prenylated isoflavonoid, kievitone. The bean pathogen, *Fusarium solani* f. sp. *phaseoli*, secretes a glycoenzyme, kievitone hydratase (EC 4.2.1.95), which catalyzes conversion of kievitone to a less toxic metabolite. Among *F. solani* strains, those that are highly virulent to *P. vulgaris* also produce kievitone hydratase constitutively, suggesting that the enzyme is a virulence factor. Based on the N-terminal amino acid sequence of purified enzyme, the kievitone hydratase cDNA and gene (*khs*) were cloned. The identities of *khs* and the cDNA were confirmed by their expression in transgenic *Neurospora crassa* and *Emericella nidulans*. Based on the gene and cDNA sequences, *khs* is predicted to encode a preprotein of 350 amino acids, from which a 19 amino acid N-terminal transit peptide is removed during maturation and secretion. The predicted mass of the mature polypeptide, 37 kDa, contrasts with the 47 to 49 kDa size estimated by electrophoresis of purified enzyme, confirming that the enzyme is extensively glycosylated. The inferred polypeptide sequence has seven canonical sites for *N*-glycosylation. Southern blot-hybridization analysis of *F. s.* f. sp. *phaseoli* DNA indicates one *khs* locus and an additonal locus with weak hybridization to the *khs* probe. Sequences related to *khs* were also detected in several isolates of *F. solani* and the related teleomorph, *Nectria haematococca*. However, strains of *F. oxysporum* known to exhibit inducible kievitone hydratase activity (but not pathogenic to bean) did not have detectable *khs* homology. Nevertheless, all isolates known to cause severe disease on bean possessed *khs* sequence.

Plants respond to microbial infections by undergoing substantial structural and metabolic changes. Among these responses is de novo synthesis of antimicrobial compounds termed phytoalexins (Müller 1956; Paxton 1981). In many systems, phytoalexins are thought to play a role in plant defenses against pathogenic colonization (Dixon 1986). Therefore, it has been hypothesized that the enzymic detoxification of phytoalexins by some fungi may contribute to their abilities to establish infection (Smith and Banks 1986; VanEtten et al. 1989).

Corresponding author: C. L. Schardl; E-mail: clscha00@ukcc.uky.edu

Fusarium solani (Mart.) Sacc. f. sp. *phaseoli* (Burk.) Snyd. *et* Hans., the causative agent of foot rot of *Phaseolus vulgaris* L. (French bean), can metabolize several of its host's isoflavonoid phytoalexins including kievitone, phaseollin, phaseollidin, and phaseollinisoflavan (Smith et al. 1981; Zhang and Smith 1983). Kievitone is an abundant isoflavonoid produced by *P. vulgaris* soon after microbial infection. The detoxification of kievitone by *F. s.* f. sp. *phaseoli* results from the action of the predominantly secreted enzyme, kievitone hydratase (EC 4.2.1.95), which catalyzes the addition of the elements of water to the dimethylallyl moiety of the substrate (Kuhn and Smith 1979; Turbek et al. 1990). Among *forma speciales* of *F. solani* there is a correlation between the constitutive production of kievitone hydratase and virulence on *P. vulgaris* (Smith et al. 1982). All isolates found to be highly virulent to *P. vulgaris* constitutively produced kievitone hydratase.

The kievitone hydratase activity of *F. s.* f. sp. *phaseoli* isolate FB1-S is attributable to a secreted, acidic glycoprotein which is active and highly stable in cell-free culture filtrate (Cleveland and Smith 1983). The enzyme has been purified from cultures of *F. s.* f. sp. *phaseoli* strain FB1-S, and is detected as two species with molecular sizes of 47 and 49 kDa, as estimated by gel electrophoresis. The two species share at least the same N-terminal sequence, and it is possible that they differ only in their degree or nature of glycosylation (Turbek et al. 1990). Kievitone hydratase activity copurified with a low level of the related activity, phaseollidin hydratase, but partial separation of these activities by non-denaturing gel electrophoresis indicated that they were different enzymes (Turbek et al. 1992).

Here we report the cloning of the *F. s.* f. sp. *phaseoli* gene, *khs*. We demonstrate by its expression in other fungal species that *khs* encodes kievitone hydratase, but not phaseollidin hydratase. In addition, a survey of other strains of *F. solani* and *Fusarium oxysporum* Schlechtend.:Fr. with various abilities to produce kievitone hydratase activity indicates that all strains highly pathogenic to *P. vulgaris* have *khs* homology.

RESULTS

Cloning the kievitone hydratase gene (*khs*) by polymerase chain reaction.

Two degenerate oligonucleotide mixtures, log numbers 1461 and 2803 (Table 1), were designed based on the N-terminal sequence of purified kievitone hydratase, and were

used for mixed oligonucleotide-primed amplification of cDNA (MOPAC) (Griffin et al. 1989) derived from *F. s.* f. sp. *phaseoli* mRNA. Six independent clones of the MOPAC products were sequenced. All were 68 bp in length, as expected, and their sequences were colinear and mostly in agreement with the kievitone hydratase N-terminal sequence previously reported (Turbek et al. 1990). Of the three differences between the reported peptide sequence and that predicted from MOPAC products, two (amino acid positions 34 and 37 in Fig. 1) had previously been considered uncertain. The third (amino acid 32) had been reported as leucine, but sequences of the MOPAC products indicated that the residue was aspartate. Reexamination of the peptide sequencing profile also indicated that amino acid 32 was more likely to be aspartate (data not shown).

Based on a consensus of the MOPAC fragment sequences, a fourfold degenerate oligonucleotide (primer number 2925) was synthesized and used with the linker-primer (Table 1) for anchored PCR of cDNA. Products larger than 1,000 bp were cloned. The cloned insert in pKAES122 was sequenced in its entirety, and spanned the region from nucleotide position 61 to 1233 (minus the intron) in Figure 1.

A segment of cDNA amplified from pKAES122 was used to probe a lambda phage library of *F. s.* f. sp. *phaseoli* cDNA. Two phage clones were identified and sequenced. One clone, pKAES123, had an insert of 1.4 kb of which 1,241 bp was later determined to be *khs* cDNA (Fig. 1), corresponding to the size of the homologous mRNA estimated by northern blot analysis (data not shown). The other cDNA clone, pKAES124, contained an insert of 0.95 kb, lacking sequence from the 5′ end of *khs* mRNA. Neither cDNA clone had the expected 5′-end linkage to the vector, and pKAES123 had a short, unrelated cDNA fragment ligated head-to-head with *khs* cDNA. The *khs* cDNA sequences in pKAES123 and pKAES124 were identical to that of pKAES122.

The cDNA insert in pKAES123 had, within its 5′ region, two potential ATG start codons in tandem that were colinear with the N-terminal sequence of mature kievitone hydratase (Fig. 1). Both of the tandem ATG codons were in contexts typical of eukaryote start codons (Kozak 1989). Assuming that the first is the actual start codon, the inferred polypeptide included a sequence of 19 N-terminal amino acid residues that was not present in the mature enzyme and, therefore, was the likely transit peptide. The mature kievitone hydratase polypeptide was predicted to be 331 amino acids in length, with a mass of 37,175 daltons.

A portion of the *khs* gene from genomic DNA was amplified by a single-primer polymerase chain reaction (SSP-PCR) using primer number 3614 (Table 1). The 2.3-kb amplified DNA was cloned to produce plasmid pKAES125 (Fig. 2). Sequence analysis indicated that it spanned the 5′ portion of *khs* to codon 163 and included a 64 bp intron and more than 1,100 bp of 5′-flanking DNA. This fragment was cloned to generate pKAES126. SSP-PCR of genomic DNA with primer 3404 gave a 1.9 kb fragment extending downstream from position 421 (codon 110) of *khs*. A total of 1,025 bp of the cloned fragment was sequenced, spanning and verifying the sequences at the 3′ termini of the cDNA clones.

Comparison of the cDNA sequences with those of the amplified genomic DNAs indicated that one 64-bp intron interrupted the *khs* open reading frame between codons 38 and 39 (Fig. 1). Also, the 5′ end of the longest cDNA clone (pKAES123) was 26 nucleotides upstream of the first ATG triplet, which was in frame with the MOPAC product and colinear with the N-terminal protein sequence. Finally, in the genomic region upstream of the cDNA sequence, and 90 to 85 bp upstream of the putative start codon, was a TATAA sequence that may have been involved in promoting transcription.

Confirmation of khs clones by heterologous expression.

To test whether the cloned cDNA and homologous genomic DNA encoded functional kievitone hydratase, a 3.3-kb fragment spanning the entire gene (Fig. 2) was amplified by PCR and introduced by cotransformation with selectable markers (see Materials and Methods) into two ascomycetous fungi, *Emericella nidulans* (Eidam) Vuill. (*Aspergillus nidulellus* R.A. Samson *et.* W. Gams) and *Neurospora crassa* Sheer *et*

Table 1. Oligonucleotides in this study[a]

Primer designation	Sequence	Locus (positions)	Features
M13-20	5′-GTAAAACGACGGCCAGT-3′	pBluescriptKS	flanks *Sac*I site
T3	5′-CGGAATTAACCCTCACTAAAG-3′	pBluescriptKS	flanks *Kpn*I site
SK	5′-CGCTCTAGAACTAGTGGATC–3′	pBluescriptKS	flanks *Sma*I site
Linker-primer	5′-$(GA)_{10}$ACTAGTCTCGAG$(T)_{18}$-3′	mRNA poly-A tail	has *Xho*I site
1461	5′-AARCAYCCNAARCARTAY-3′	*khs* (61–78)	128 X degenerate
2803	5′-ACNGG(R/T)ATRTCNCCRTT-3′	*khs* (192–179, 114–112)	192 X degenerate
2925	5′-AARCATCCGAARCAGTAC-3′	*khs* (61–78)	4 X degenerate
3199	5′-AC(G/C)GGGATGTC(R/C)CCATT-3′	*khs* (192–179, 114–112)	6 X degenerate
3404	5′-GTTGGTGATAATGGAATC-3′	*khs* (422–439)	
3405	5′-GTCAAGGATGCTGCTAA-3′	*khs* (979–963)	
3613	5′-GGACCTACCAGTCTACCGCG-3′	*khs* (882–901)	
3614	5′-CCAGCATTAGAAATGACCCG-3′	*khs* (549–530)	
3878	5′-AACAGACGAGATCATCATTTTCTCGGTTACTTG-3′	*khs* (18–1), *tub2* (-1–-15)	
3879	5′-ATGATGATCTCGTCTGTT-3′	*khs* (1–18)	
4221	5′-GTACTGTTTCGGATGCTTG-3′	*khs* (78–60)	
4222	5′-CAGGGTCACATCACAAAG-3′	*khs* (-987–-970)	
4223	5′-CTTCCTCGAGGACCACGATATTCCGCAG-3′	*khs* (*ca.* 2,300)	has *Xho*I site
4347	5′-CACATGGTAGCATGGGAT-3′	*khs* (-474–-467)	
4348	5′-CTTGAGCTGGCACAGCTA-3′	*khs* (1190–2007)	

[a] Code: A = dAMP, T = dTMP, C = dCMP, G = dGMP, R = dAMP, or dGMP, Y = dTMP, or dCMP, N = any nucleotide, and other degeneracies are in parentheses.

Dodge, fungi which did not otherwise produce detectable kievitone hydratase. Also introduced into these fungi was pKAES129 (Fig. 2), a clone of a chimeric gene containing the *khs* reading frame downstream of the *tub2* promoter of *Epichloë typhina* (Pers.:Fr.) Tul. (Tsai et al. 1992). Pools of 10 to 20 transformants from each experiment were cultured and assayed for kievitone hydratase activity. All *E. nidulans* and most *N. crassa* pooled cultures converted kievitone to kievitone hydrate (data not shown). Activity was conferred both by the amplified genomic fragment and by pKAES129 (data not shown). Furthermore, three of four clones of the amplified 3.3-kb genomic fragment conferred to transformed *E. nidulans* the ability to produce kievitone hydratase. In no instance was activity detected from untransformed *E. nidulans* or *N. crassa*, or following transformation with only pKBY-2 or pCSN43, respectively.

Eight *N. crassa* cotransformants were selected from a pool exhibiting a high level of kievitone hydratase activity, and were individually assayed for extracellular activity. Four of the transformants converted kievitone to kievitone hydrate in

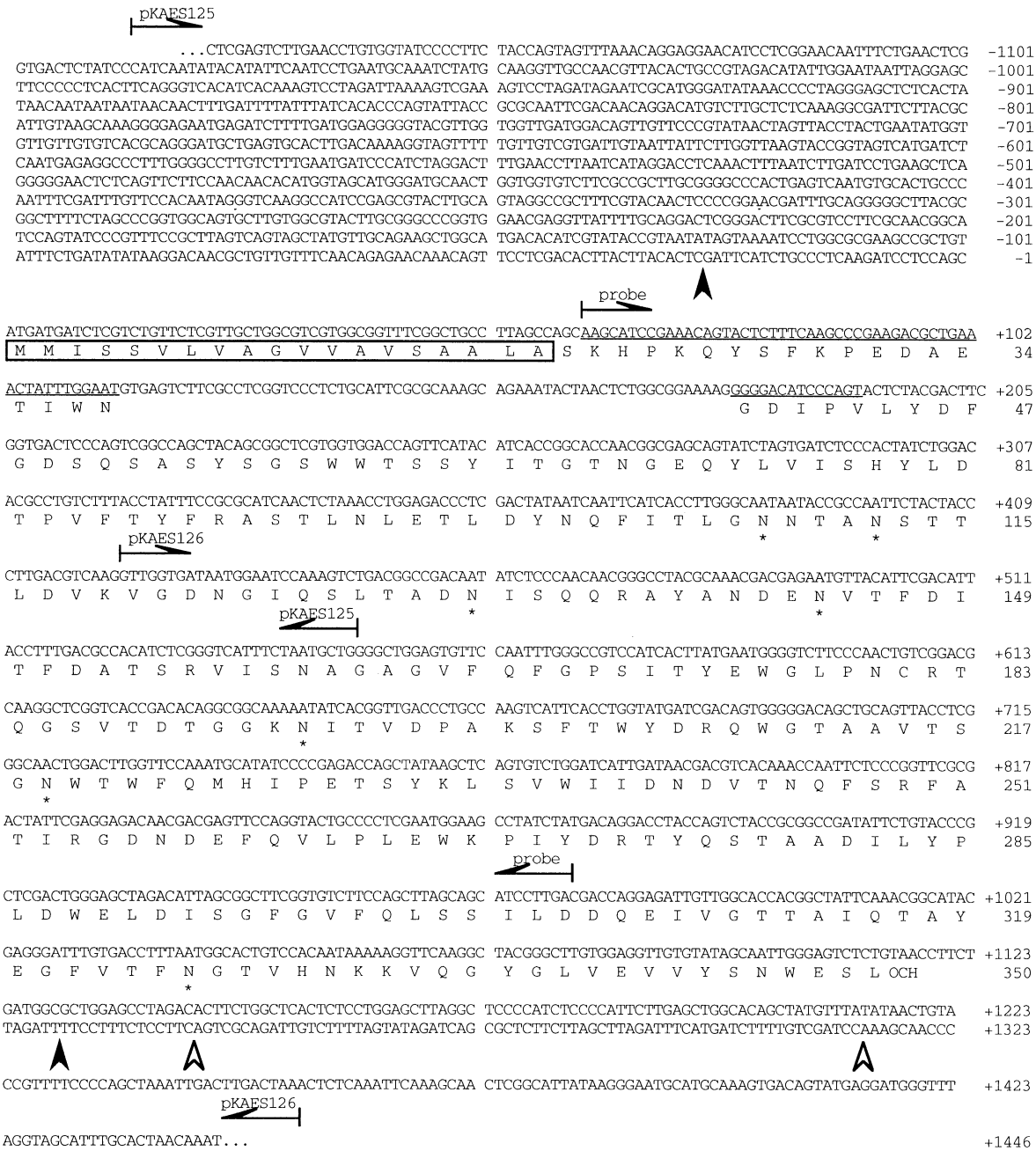

Fig. 1. Sequence of *khs* and the predicted polypeptide sequence, assuming an AUG start codon and the universal genetic code. The two filled vertical arrows bracket the *khs* cDNA insert in lambda phage clone pKAES123, which also lacked the intron sequence (positions +115 to +178). Open vertical arrows are the 3′ ends of two other cDNA clones. The nucleotide sequence corresponding to the MOPAC products is underlined. The boxed amino acid sequence is the inferred transit peptide. Asterisks (*) identify the seven putative *N*-linked glycosylation sites. The three pairs of horizontal arrows indicate three genomic DNA fragments amplified by PCR and used as "probe" or cloned in the plasmids indicated.

amounts that were spectrophotometrically verifiable (Table 2). Two other transformants produced trace activity (Fig. 3, lanes 7 and 10). Untransformed *N. crassa* (lane 1) and a transformant that received only pCSN43 (lane 2) showed no kievitone hydratase activity. Six of twelve selected *E. nidulans* transformants obtained after cotransformation with the 3.3-kb PCR fragment and pKBY-2 also produced and secreted substantial kievitone hydratase activity (Fig. 4, lanes 5, 6, 8, 10, 11, 14; Table 2), and two more produced trace activity (Fig. 4, lanes 7 and 9). Southern blot hybridization indicated that most *N. crassa* (Fig. 3) and *E. nidulans* (Fig. 4) transformants expressing kievitone hydratase activity had multiple copies of *khs*.

There was considerable variation in kievitone hydratase activity among transformants (Table 2). The reasons for this variation were unknown, but Southern blot hybridization analysis (Figs. 3 and 4) suggested that enzyme activity levels were not simply a function of the *khs* gene copy number in each transformant.

The two transformants each of *E. nidulans* and *N. crassa* expressing the highest level of secreted kievitone hydratase activity were assayed for phaseollidin hydratase. Kievitone was included in each assay so that its conversion to kievitone hydrate served as an internal, positive control. After an extended reaction time of 14 h, the culture filtrates completely converted kievitone to kievitone hydrate, but no phaseollidin hydrate was detected and the phaseollidin was recovered from the reaction mixtures (data not shown). These results demonstrated that the enzyme encoded by *khs* had kievitone hydratase activity but no phaseollidin hydratase activity.

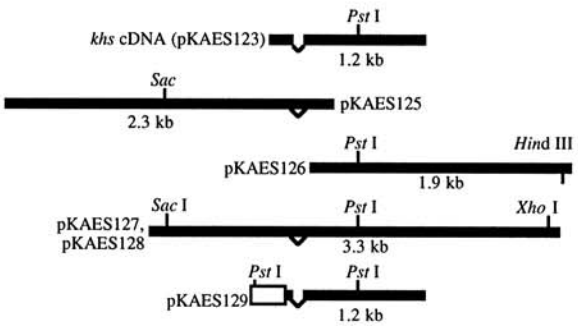

Fig. 2. Maps and relative positions of cloned *khs* cDNA (pKAES123), genomic DNA (pKAES125, pKAES126, pKAES127, and pKAES128) and the *tub2* promoter-*khs* fusion in pKAES129. Black bars indicate *Fusarium solani* f. sp. *phaseoli* DNA sequence. The intron position is indicated by a V beneath each bar. The open box in the pKAES129 map is the 5′ region, including the promoter, of the *tub2* gene of *Epichloë typhina*.

Table 2. Secreted kievitone hydratase activities of fungal transformants[a]

Transformant number	KHase activity[b]
Neurospora crassa	
1 (untransformed)	Undetectable
2 (pCSN43 only)	Undetectable
4[c]	0.11 ± 0.07
6[c]	2.70 ± 1.60
8	0.03 ± 0.01
9	0.01 ± 0.01
Emericella nidulans	
1 (untransformed)	Undetectable
2 (pKBY-2 only)	Undetectable
5	11.4 ±2.40
6	9.60 ±3.50
8[c]	20.0 ± 5.60
10	1.10 ±0.24
11[c]	15.9 ± 5.60
14	1.50 ±0.29

[a] Transformant numbers correspond to lane numbers in Fig. 3 (*N. crassa*) and Fig. 4 (*E. nidulans*). Except where otherwise indicated, transformants received either pCSN43 (*N. crassa*) or pKBY-2 (*E. nidulans*) and the 3.3 kb amplified genomic fragment from the *F. solani* FB1-S *khs* locus.

[b] Results represent mean value and standard deviation of five replicates. Activity is expresed as units/mg mycelial dry wt, where one unit catalyzed the production of one nmol kievitone hydrate per minute at 27°C.

[c] Transformants also assayed for phaseollidin hydratase activity.

Sequences homologous to khs in *Fusarium* species.

Low-stringency Southern blot hybridization analysis of genomic DNA from *F. s.* f. sp. *phaseoli* FB1-S suggested two loci with sequence relationships to *khs* (Fig. 5). The restriction endonucleases *Bgl*II (lane 1), *Hin*dIII (lane 3), and *Xho*I (lane 4) do not cut within the DNA fragment used as probe, so detection of more than two bands in each of these lanes indicated the presence of more than one *khs*-related sequence in the genome of *F. s.* f. sp. *phaseoli*. Following digestion

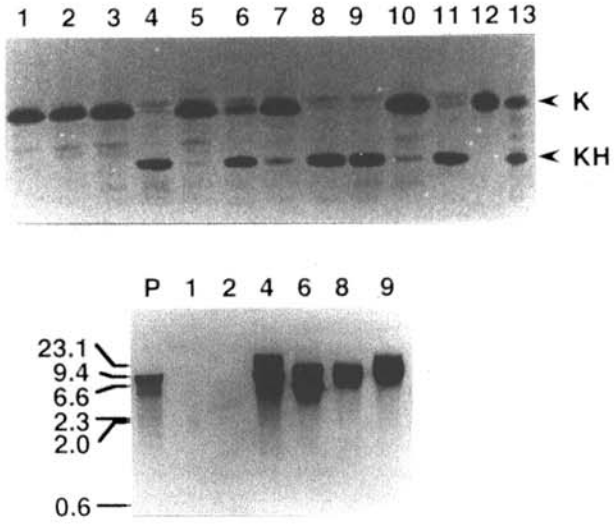

Fig. 3. Thin-layer chromatogram demonstrating conversion of kievitone to kievitone hydrate by culture filtrates from *N. crassa* transformants (upper panel), and Southern blot analysis of *khs* in selected transformants (lower panel). Upper panel: 100 µg of kievitone was added to 5 ml of culture filtrate of untransformed *N. crassa* (lane 1), *N. crassa* transformed with only pCSN43 (lane 2), and eight hygromycin resistant isolates from a cotransformation experiment using pCSN43 and the amplified 3.3-kb genomic DNA fragment spanning *khs* (lanes 3 to 10). The sample in lane 11 is from a reaction with 30 units of partially-purified kievitone hydratase from *F. solani* FB1-S; lanes 12 and 13 contained kievitone (K) and kievitone hydrate (KH) standards. The chromatogram was stained with diazotized *p*-nitroaniline (Stahl 1965). Quantitative assay results are presented in Table 2. Lower panel: Southern blot of selected DNA from *N. crassa* probed with a *khs* cDNA fragment (codons 21 to 306). Lane numbers correspond to those in the upper panel; lane P contains DNA from *F. solani* FB1-S. Each lane contained 10 µg of genomic DNA cleaved with *Sac*I and *Xho*I. Positions of the size markers—*Hin*dIII fragments of bacteriophage lambda DNA—are indicated at left.

with *Eco*RI (lane 2), which also did not cut within the *khs* sequence, only one strong band was observed. The possible explanations are that the related sequences were closely linked and present on the same *Eco*RI fragment, that the *Eco*RI fragments from the two loci had similar size, or that the *khs*-related sequence was cut into small fragments which were undetected. High-stringency washes consistently rendered faint or undetectable one of the bands in each digest, leaving only the stronger band, and suggesting that *F. s.* f. sp. *phaseoli* FB1-S contains only a single copy highly homologous to *khs*.

Other strains of *F. solani*, *Nectria haematococca* Berk. *et* Br., and *F. oxysporum* were surveyed for sequences related to *khs* by Southern blot hybridization to *Xho*I-cleaved total DNA (Table 3). Bands were observed from eight of the 13 strains (including *F. s.* f. sp. *phaseoli* FB1-S) known to produce kievitone hydratase activity. Genomic DNAs of *F. s.* f. sp. *phaseoli* isolates W8-BK and W9-BK, and of *F. solani* strain A (Roy et al. 1989) isolate F17-R, gave similar hybridization patterns to that of *F. s.* f. sp. *phaseoli* FB1-S following both low-stringency and high-stringency washes. Under low-stringency conditions, two *Xho*I bands were detected from *F. s.* f. sp. *phaseoli* S136-T, an isolate which causes mild foot rot on bean and produces kievitone hydratase (Smith et al. 1982). Both bands from S136-T were nearly undetectable following the high-stringency wash, suggesting that strain S136-T had one or two *khs*-related loci with substantial difference from FB1-S *khs* sequence. *N. haematococca* MP VI isolate T8-V, which causes slight foot rot on bean (Smith et al. 1982), had a hybridization pattern similar to that of S136-T. Of the five isolates analyzed that did not produce detectable kievitone hydratase (Smith et al. 1982), only isolate T231-V of *N. haematococca* MP VI had a *khs*-related sequence. Surprisingly, *F. oxysporum* isolates B1-GK and B6-GK had no detectable homology to *khs* (Table 3), though they exhibit inducible kievitone hydratase activity (Smith et al. 1982).

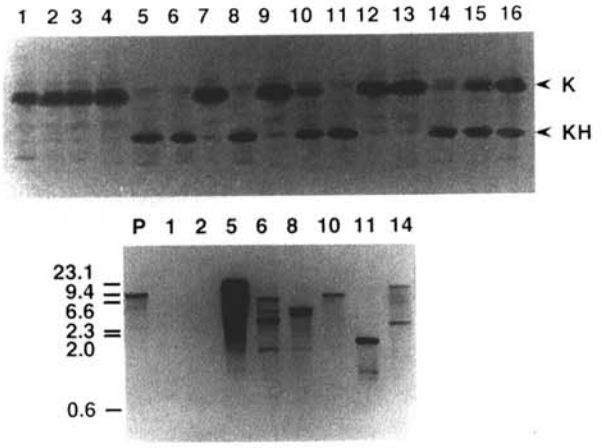

Fig. 4. Thin-layer chromatogram demonstrating conversion of kievitone to kievitone hydrate by culture filtrates from *E. nidulans* transformants (upper panel), and Southern blot analysis of *khs* in selected transformants (lower panel). Upper panel: 100 µg of kievitone was added to 5 ml of culture filtrate of untransformed *E. nidulans* (lane 1), *E. nidulans* transformed with only pKBY-2 (lane 2), and 12 tryptophan autotrophic isolates from a cotransformation experiment using pKBY-2 and the amplified 3.3-kb genomic DNA fragment spanning *khs* (lanes 3 to 14). The sample in lane 15 is from a reaction with 30 units of partially purified kievitone hydratase from *F. solani* FB1-S; lane 16 contained kievitone (K) and kievitone hydrate (KH) standards. The chromatogram was stained with diazotized *p*-nitroaniline (Stahl 1965). Quantitative assay results are presented in Table 2. Lower panel: Southern blot of selected DNA from *E. nidulans* probed with a *khs* cDNA fragment. Lane numbers correspond to those in the upper panel; lane P contains DNA from *F. solani* FB1-S. Each lane contained 10 µg of genomic DNA cleaved with *Sac*I and *Xho*I. Positions of the size markers (as in Fig. 3) are indicated at left.

DISCUSSION

Kievitone hydratase, produced and secreted by *F. s.* f. sp. *phaseoli* strain FB1-S, is the first phytoalexin-detoxifying enzyme to have been purified to near homogeneity and for which N-terminal sequence data have been obtained (Turbek et al. 1990). These data facilitated the isolation, by variations on PCR, of a cDNA copy of the mRNA, and of the complete *khs* gene encoding this enzyme. To eliminate the possibility that the peptide sequence was derived from a copurified protein rather than from the enzyme of interest, a functional assay was used to confirm the identity of the cloned *khs* and cDNA. As was the case for pisatin demethylase (Weltring et al. 1988), kievitone hydratase was not detected in untransformed *E. nidulans*, a well-characterized ascomycete for which DNA-mediated transformation is routine (Yelton et al. 1984; Yelton et al. 1985). Therefore, heterologous expression of *khs* in this fungus, as well as in *N. crassa*, provided a convenient method to confirm the identity of the gene.

The levels of kievitone hydratase activity secreted by *E. nidulans* and *N. crassa* transformed with cloned *khs* were generally comparable to, or much higher than, those obtained from *F. s.* f. sp. *phaseoli* FB1-S (Choi et al. 1987; Turbek et

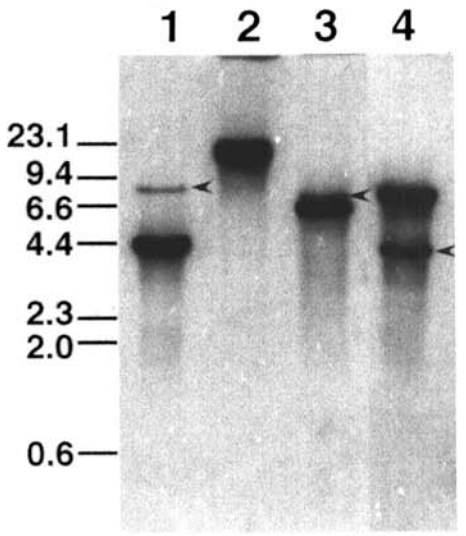

Fig. 5. Detection of *khs*-homologous sequences in genomic DNA of *F. solani* f. sp. *phaseoli*. Samples of 10 µg of total genomic DNA were digested completely with restriction endonucleases *Bgl*II (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), and *Xho*I (lane 4). The DNA samples were fractionated by electrophoresis in 0.8% HGT agarose gel, then transferred onto GeneScreen Plus membrane. The membrane was hybridized with a 857-bp digoxigenin-labeled probe spanning codons 21 to 306 of *khs* cDNA. The filter was washed at low stringency. Arrows indicate bands that were faint or absent following high-stringency wash (data not shown). Positions of size markers are indicated at left. Markers were generated by *Hin*dIII digestion of DNA of bacteriophage lambda.

al. 1990). Compared with the uninduced activities from *F. s.* f. sp. *phaseoli*, positive *E. nidulans* transformants produced 30- to 570-fold higher activities, and *N. crassa* transformants produced up to 135-fold higher levels. These results may be explained, in part, by integration of multiple copies of *khs* into the genomes of the transformed fungi. However, the levels of secreted kievitone hydratase among transformants were not directly proportional to the number of integrated *khs* copies. It is also possible that *khs* expression is somewhat repressed in *F. s.* f. sp. *phaseoli* and that, when *khs* is transferred to the heterologous fungal genomes, it is not subject to such regulation.

F. s. f. sp. *phaseoli* carries a single copy of *khs* as determined by Southern blot hybridization analysis (Fig. 5). Another locus was detected only at low washing stringency, indicating that it has only partial or low sequence homology to *khs*. This weakly hybridizing fragment may represent another gene encoding a kievitone hydratase enzyme, but with low homology to the cloned *khs* gene; that is, it may encode an isozyme. Alternatively, the weak band may represent a *khs* pseudogene (i.e., nonfunctional). A third possibility is that this band represents a gene encoding an enzyme, such as phaseollidin hydratase, with an activity related to that of kievitone hydratase (Kuhn and Smith 1979; Turbek et al. 1992).

Kievitone hydratase is secreted and modified by glycosylation and, very likely, by cleavage of a signal peptide. Previous analysis (Turbek et al. 1990) indicated that the N-terminus of mature kievitone hydratase is the serine residue corresponding to codon 20 (Fig. 1). The *khs* gene and cDNA sequences indicate that this residue is preceded in the preprotein by a 19-amino acid N-terminal portion. There is a possibility that the cDNA clone was not near full length, and that the true signal peptide of kievitone hydratase preprotein was actually much larger. This is unlikely, however, because pKAES129 directed secretion of the active enzyme. This plasmid had a promoter fusion constructed so that translation of the kievitone hydratase coding region would begin with the 19-mino acid putative signal. The fact that *E. nidulans* transformed with pKAES129 secreted the enzyme into the culture medium indicated that the 19-amino acid N-terminus was sufficient for secretion.

Kievitone hydratase secretion is consistent with the model proposed by Blobel and Dobberstein (1975). Proteolytic processing is usually associated with transport of proteins into the endoplasmic reticulum and with secretion. However, the site of cleavage is known for only a small proportion of the known secreted proteins in various eukaryotic systems. Most transit peptide-cleavage sites are inferred from the rules of von Heijne (1986) and have not been identified experimentally. Therefore, it is significant that the features of the transit peptide and the cleavage site in the kievitone hydratase preprotein are largely in keeping with these rules, though the predicted kievitone hydratase preprotein does not include basic amino acids in its N-terminal region.

In several host–fungus pathosystems involving *F. solani* strains and legumes (Fabaceae), the pathogens are able to enzymically modify the isoflavonoid compounds which are produced by their hosts as apparent defensive factors (Lucy et al. 1988; Miao and VanEtten 1992; Smith and Banks 1986; VanEtten et al. 1989). In this paper, the correlation was extended in the *F. solani–P. vulgaris* pathosystem by the observation of *khs* sequences in all *F. solani* isolates surveyed that are known to be highly virulent to *P. vulgaris*. However, kievitone hydratase activity has also been observed in strains of *F. solani* and *F. oxysporum* not known to parasitize legumes, and for which the enzyme has no obvious role (Smith et al. 1982). Here it is reported that *khs*-related sequences and the corresponding enzyme activity occurs in several *F. solani* strains that are not pathogenic on *P. vulgaris*. Therefore, the

Table 3. Detection of *khs*-homologous sequences from *Fusarium solani*, *Fusarium oxysporum*, and *Nectria haematoccoca*

Species	Isolate[a]	Hybridizing *Xho*I fragments detected (kbp)		Kievitone hydratase[b]
		Low stringency	High stringency	
F. solani f. sp. *phaseoli*	FB1-S*	4.3, 7.8	7.8	+
	S136-T*	5.2, 8.6	None	+
	W8-BK*	4.3, 7.8	7.8	+
	W9-BK*	4.3, 7.8	7.8	+
F. solani	S333-T	4.3, 15	15	+
F. solani f. sp. *cucurbitae* race 2	S198-T	None	None	–
F. solani strain A[c]	F17-R*	4.3, 7.8	7.8	+
N. haematococca MP VI	T8-V	5.2, 8.6	None	+
	T27-V	5.2, 8.6	5.2, 8.6	+
	T30-V	None	None	+
	T86-V	None	None	–
	T111-V	None	None	–
	T200-V	None	None	(+)
	T224-V	None	None	(+)
	T231-V	8.6	8.6	–
F. oxysporum f. sp. *cucumerinum*	B1-GK	None	None	+
F. oxysporum f. sp. *melonis*	B6-GK	None	None	+
F. oxysporum f. sp. *lycopersici*	M811-GK	None	None	–

[a] Isolate codes precede hyphens; letters following hyphens designate sources: S = D. A. S.; T = Tousson, Pennsylvania State University; BK = Burke and Kraft, USDA, Washington State University; GK = Gessler and Kuc, University of Kentucky; R = Rupe, University of Arkansas; V = VanEtten, University of Arizona. Asterisks (*) known or suspected pathogens of *P. vulgaris*.

[b] Data from Smith et al. 1982. Abbreviations are + = activity detected after 24 h substrate induction, (+) = trace activity after induction, – = no detectable activity.

[c] Roy et al. 1989.

presence and activity of genes for phytoalexin detoxification, though perhaps important for virulence of the pathogen or its competitiveness in host colonization, are not sufficient to confer virulence.

Results from studies of *Fusarium solani* f. sp. *pisi* (F.R. Jones) Snyder *et* Hans. (teleomorph = *N. haematococca* MP VI) strains are somewhat analogous. In this system the enzyme activity, pisatin demethylase (PDA), catalyzes oxidative demethylation of the pea phytoalexin, pisatin. Those strains that are highly virulent to pea (*Pisum sativum*) produce PDA (VanEtten et al. 1989). Considerable variation is observed among isolates in both the induced activity levels of the enzyme (Mackintosh et al. 1989) and in the degree of detectable homology to the gene, *PdaT9*. Homologous sequences are generally present in strains that exhibit a high level of virulence on pea and produce high levels of PDA following substrate induction (Miao et al. 1991b). A strong genetic linkage of PDA, metabolism of pisatin, and virulence on pea has been observed (Tegtmeier and VanEtten 1982). However, recent molecular genetic and karyotype studies have indicated that the *PdaT9* gene is on a dispensible (B) chromosome (Miao et al. 1991a). The B chromosome probably possesses other virulence determinants, explaining the genetic linkage of virulence to *PdaT9* when PDA-minus parents lacked its homolog. Nevertheless, there is evidence of a role of pisatin demethylase in virulence. Acquisition of *PdaT9* by transformation converted the maize pathogen, *Cochliobolus heterostrophus* (Drechs.) Drechs., into a weak pea pathogen (Schäfer et al. 1989). Furthermore, though gene disruption mutants of *N. haematococca* are still pathogenic, preliminary tests suggest their virulence may be reduced (H. D. VanEtten, personal commmunication). Strains of *N. haematococca* that produced PDA possessed sequences that hybridized to the *PdaT9* probe, whether or not they were virulent to pea (Mackintosh et al. 1989; Reimmann and VanEtten 1994). Thus, like kievitone hydratase in the *F. solani*–bean pathosystem, pisatin demethylase activity may contribute to virulence but, if so, is very unlikely to be the sole virulence determinant.

The origins of genes for phytoalexin tolerance are of interest with respect to the evolution of plant pathogens. The existence of kievitone hydratase in strains whose hosts are not known to produce isoflavonoid defense compounds, and which do not cause disease on bean, indicates that this enzyme alone is not sufficient to confer virulence. Other isoflavonoid-detoxifying enzymes may be needed, along with additional compatibility and recognition factors. Furthermore, it is overly simplistic to infer that the presence of *khs* is the direct result of evolution for compatibility with *P. vulgaris* or a related host. The distribution of *khs* among strains may partly be explained by sexual and, perhaps, parasexual transfer (Glass and Kuldau 1992), or the genes may be relics from a common ancestor which required them to parasitize its host or hosts. These conjectures seem reasonable for cases in which *khs* or *khs*-related sequences were observed in several *forma speciales* of *F. solani*. However, they may not explain the production of kievitone hydratase by strains that have no detectable *khs* homology. In these instances it may be that kievitone hydratase activity is attributable to enzymes with different functions, such as hydrolysis of cell wall components or proteolysis, which also involve addition of water to substrate molecules. Alternatively, the amino acid sequence of the enzyme or the gene sequence may be poorly conserved over evolutionary time.

MATERIALS AND METHODS

Biological materials.

F. s. f. sp. *phaseoli*, isolate FB1-S (ATCC 60860), is a pathogen of *Phaseolus vulgaris* L. (French bean) and was the source of purified kievitone hydratase (Turbek et al. 1990). This and other fungal strains in this study were characterized previously (Choi et al. 1987; Smith et al. 1982). Isolates of *F. solani* strain A, the soybean sudden death syndrome pathogen (Roy et al. 1989; Rupe 1989), were supplied by J. C. Rupe (University of Arkansas, Fayetteville). All *Fusarium* and *Nectria* strains were grown as previously described (Smith et al. 1982). *Emericella nidulans* strain UCD-1 (Yelton et al. 1984), and the wild-type *Neurospora crassa* strain OR23-IVA were obtained from the Fungal Genetics Stock Center (Manhattan, Kansas).

Nucleic acids and primers.

All oligodeoxyribonucleotides used as primers for PCR are listed in Table 1, and were synthesized in the University of Kentucky Macromolecular Structure Analysis Facility using an Applied Biosystems (Foster City, Calif.) 380B DNA synthesizer.

Total RNA was prepared as previously described (Choi et al. 1990), with modifications. A culture of *F. s.* f. sp. *phaseoli* FB1-S was first treated with biochanin A for 9 h (Turbek et al. 1990). After the RNA was pelleted by ultracentrifugation it was washed by vortexing twice each with 1 ml of 60 mM sodium acetate (pH 7.0) in 70% 2-propanol to remove excess CsCl (Berger and Chirgwin 1989). Each pellet was then dissolved in 1 ml of 10 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, and 1% sodium lauryl sulfate (SDS) by freezing at –70°C for 30 min and thawing at ambient temperature.

Fungal genomic DNA was isolated as previously described (Choi et al. 1990).

cDNA library construction and screening:

Poly(A)+ RNA was isolated from total *F. s.* f. sp. *phaseoli* RNA by oligothymidylic column chromatography (Aviv and Leder 1972) and used for construction of a cDNA library in lambda phage Uni-ZAP (Stratagene Cloning Systems, La Jolla, Calif.) (Short et al. 1988). Plaques were grown on a lawn of *Escherichia coli* PLK-F′ cells at a density of 1,500 pfu per 150-mm plate, then transferred to uncharged nylon membranes (Stratagene) and screened with a digoxigenin-labeled probe (Gebeyechu et al. 1987). Positive plaques were confirmed by PCR using primer numbers 2925 and 3405 (Table 1). For purification, plaques were eluted in SM buffer, diluted, replated and rescreened (Ausubel et al. 1987–1994).

Polymerase chain reactions and sequence determination.

Deoxyribonucleotide primers for PCR and sequence analysis are listed in Table 1. Unless otherwise specified, PCR buffer contained 67 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.8, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 16.6 mM $(NH_4)_2SO_4$, 6.7 μM

ethylenediaminetetraacetic acid (EDTA), 0.17 μg μl^{-1} bovine serum albumin (BSA), 10% (v/v) dimethyl sulfoxide (DMSO), and 1.5 mM each of dGTP, dATP, dTTP, and dCTP (Griffin et al. 1989). Standard temperature regimes were 95°C for 2 min, followed by 40 cycles of 95°C for 1 min, 52°C for 2 min, 72°C for 2 min. In most cases, PCR products were cloned into dT-tailed pBluescript KS(+) or pBluescript KS(–) (Stratagene) prepared by the method of Marchuk et al. (1991). Recombinant plasmids were introduced by transformation (Hanahan 1983) into *Escherichia coli* XL1-Blue (Bullock et al. 1987). Single-stranded plasmid DNAs were prepared using helper phage M13K07 (Vieira and Messing 1987). DNA sequences were determined for both strands by the Sanger random chain termination method using Sequenase modified T7 DNA polymerase (United States Biochemical, Cleveland, Ohio) (Toneguzzo et al. 1988) and primers described in Table 1.

For mixed oligonucleotide-primed amplification of cDNA (MOPAC) (Griffin et al. 1989), a 100-μl reaction mixture included 1.8 nmol each of primers 1461 and 2803, 150 ng of double-stranded cDNA, 2.5 units of *Taq* DNA polymerase (U.S. Biochemicals) and other components described above. The following profile was run for 40 cycles: denaturation at 95°C for 1 min, annealing at 46°C for 2 min, and extension at 72°C for 1 min. The reactions were then incubated at 72°C for 7 min and cooled to 4°C. Products were analyzed by gel electrophoresis in a composite agarose gel consisting of 3% NuSieve GTG agarose and 1% SeaKem HGT agarose (FMC Marine Colloids, Rockland, MD).

Anchored PCR of cDNA (Loh et al. 1989) was carried out in a 100-μl reaction containing 84 pmol of primer 2925, 17 pmol linker-primer, and 13.5 ng of double-stranded cDNA.

For single primer-mediated PCR (Wang et al. 1991) of the *khs* gene 5′ region, 50 μl of PCR reaction mix contained 50 mM Tris-HCl (pH 8.8), 2.5 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 2.5 mM 2-mercaptoethanol, 8.5 μg of BSA, 10% DMSO, 200 μM each dNTP, 13.4 pmol primer 3614, 40 ng of total genomic DNA and 1 unit of *Taq* DNA polymerase (Promega Corp., Madison, Wisc.).

The *khs* 3′ region was amplified by single-specific-primer PCR (SSP-PCR) (Shyamala and Ames 1989). Genomic DNA was digested with *Hin*dIII and *Bgl*II, and 0.4 μg of digested genomic DNA was ligated (Ausubel et al. 1987-1994) to 4 μg *Hin*dIII and *Bam*HI-cut pBluescriptKS(–) in a volume of 50 μl. Then, 2 μl of the ligation mixture was included in 50-μl PCR reaction mixture with 1 unit of *Taq* polymerase (Promega Corp.) and 13.4 pmol each of primer 3404 and primer T3. The PCR temperature profile was as follows: one cycle of 94°C for 3 min, 56°C for 1 min, 72°C for 2 min; nine cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 2 min; 15 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 2 min; then 15 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min. Products were checked by first cleaving with *Pst*I and *Hin*dIII and then DNA hybridization. The cleaved fragments were subsequently cloned into pBluescript KS(–) to generate pKAES126 (Fig. 2).

Construction of a chimeric tub2 5′-*khs* gene.

The 235-bp 5′ region of the *E. typhina* β-tubulin gene, *tub2* (Byrd et al. 1990), was fused by PCR (Horton et al. 1990; Tsai et al. 1992) to the first ATG codon of full-length *khs* cDNA. Plasmid pKAES004 (Byrd et al. 1990) was used as template from which the 5′ region of *tub2* was amplified using primer T3 and primer 3878. The 18 nucleotides of the 5′ end of primer 3878 were complementary to nucleotides +1 to +18 of the *khs* gene open reading frame, and the 15 nucleotides at the 3′ end of the primer were complementary to positions –1 to –15 immediately upstream of the *tub2* coding region. Plasmid pKAES123 (Fig. 2) was used as template for amplification of the kievitone hydratase coding sequence. Primer 3879, representing nucleotides +1 to +18 of the *khs* gene, was used as the sense primer. M13–20 primer was used as the anti-sense primer. PCR reactions in 25 μl employed *Taq* polymerase (Promega Corp.). Temperature conditions were standard with an annealing temperature of 58°C. PCR products were purified by centrifugal dialysis with Centricon 30 (Amicon, Beverly, Mass.) and the final retentate was adjusted to 25 μl volume. Another PCR reaction was performed using 1 μl each of the two PCR products and 10 ng each of T3 primer and M13-20 primer in a 25-μl reaction mix. The PCR products were then digested with *Xho*I and size fractionated by agarose gel electrophoresis. Then the desired band was excised from the gel, eluted with a GeneClean kit (Bio 101, La Jolla, Calif.) (Vogelstein and Gillespie 1979), and cloned into pBluescriptKS(+).

Construction of a full-length genomic *khs* clone.

A 3.3-kb DNA fragment spanning *khs* was generated by PCR using *F. s.* f. sp. *phaseoli* FB1-S genomic DNA as template, primer 4222 (downstream of a *Sac*I site) and primer 4223 (which includes the *Xho*I site upstream of *khs*). Standard PCR conditions were employed but with 2 min annealing at 58°C. The product was introduced into fungi by cotransformation, as described below. Also, after digestion with *Sac*I and *Xho*I, the product was cloned into similarly digested vector. Clones obtained were tested for activity in transgenic *E. nidulans* (see below and Results). Two active clones were obtained in pBluescriptKS(–) and designated pKAES127-1 and pKAES127-2 (a third clone was nonfunctional). One functional clone in pBluescriptKS(+) was designated pKAES128 (Fig. 2).

Transformation and cotransformation of *E. nidulans* and *N. crassa*.

Protoplasts of *E. nidulans* UCD-1 were prepared and transformed by the method of Yelton et al. (1984). For cotransformation, 20 μg of plasmid pKBY-2 (Yelton et al. 1985) was added along with either 5 μg of uncloned PCR product or 20 μg of plasmid containing cloned *khs* sequence. After the transformant colonies became visible on the selective medium they were transferred to new selective medium plates and incubated at 37°C for 72 h to allow sporulation. Conidiospores (about 1×10^7) were then transferred to 30 ml of selective liquid medium and grown for 16 h at 37°C in preparation for activity assays.

Isolation and transformation of protoplasts from *N. crassa* was by the method of Vollmer and Yanofsky (1986). Cotransformation included 20 μg of pCSN43 (provided by C. A. Staben, University of Kentucky), with the *E. coli* hygromycin B phosphotransferase gene (*hph*) modified for hygromycin selection in fungi. Along with pCSN43 was added 5 μg of uncloned PCR product or 20 μg of plasmid containing cloned

khs sequence. Transformants were selected for growth at 34°C on Vogel agar medium with 2% sorbose, 0.05% fructose, 0.05% glucose (Vollmer and Yanofsky 1986), and 150 μg ml^{-1} hygromycin B (Sigma Chemical Co., St. Louis, Missouri). After 4 days, transformant colonies were transferred to Vogel agar with 1.5% sucrose and 150 μg ml^{-1} hygromycin B and incubated first at 34°C for 12 h, then at ambient temperature for 48 h to allow sporulation. Conidiospores (about 1×10^7) were then transferred to 30 ml of Vogel liquid medium with 1.5% sucrose and 75 μg ml^{-1} hygromycin B, and grown for 36 h at 34°C in preparation for enzyme activity assays.

Kievitone hydratase and phaseollidin hydratase activity assays.

Conversion of kievitone to kievitone hydrate in fungal cultures was determined qualitatively by thin-layer chromatography as described previously (Zhang and Smith 1983). Quantitative assays of secreted kievitone hydratase and phaseollidin hydratase were as in Turbek et al. (1990, 1992). Phaseollin hydratase activity assays used 5 ml each of cell-free culture filtrate, 50 μg each of kievitone (internal positive control), and 50 μg phaseollidin, and were incubated 27°C for 14 h with shaking. Activities of these enzymes were determined to be stable over extended times (Turbek et al. 1990, 1992). Therefore, to quantitate very low kievitone hydratase activity levels, reaction times were varied from 20 to 240 min. As defined, one enzyme unit of kievitone hydratase or phaseollidin hydratase catalyzed the production of 1 nmol product per minute at 27°C (Cleveland and Smith 1983).

DNA hybridization analysis.

Restriction endonuclease cleavage and electrophoresis of DNA, and transfer to Genescreen Plus (Dupont NEN Research Products, Boston, Mass.) positively charged nylon membranes were by standard protocols (Ausubel et al. 1987–1994). Digoxigenin-labeled probes were prepared by the standard PCR protocol, but using 135 μM dTTP and 15 μM digoxigenin-dUTP (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The *khs* cDNA probe was amplified from pKAES122 by extension of primer 2925 and primer 3405, and spanned codons 21 to 306 of *khs* cDNA. The probe was hybridized to the DNA blots as described in Ausubel et al. (1987–1994) at 42°C in 6 × SSC, 5 × Denhardt's solution, 50% formamide, 0.1% SDS, 0.1% $Na_4P_2O_7$-$10H_2O$, 50 mM Tris-HCl (pH 7.5) and 50 μg ml^{-1} denatured herring sperm DNA. For low-stringency washes, the filters were placed twice in 2 × SSC, 0.1% SDS at 55°C for 30 min. High-stringency wash conditions were 0.1 × SSC, 0.1% SDS at 68°C once for 15 min, and again for 30 min. Hybridized probe was detected immunochemically (Ausubel et al. 1987–1994) using Lumi-Phos 530 (Boehringer-Mannheim Biochemicals).

ACKNOWLEDGMENTS

We wish to thank C. S. Turbek and A. D. Byrd for technical assistance. This project was supported by United States Department of Agriculture Competitive Research Grant 90-37262-5447. This is publication number 95-22-033 of the Kentucky Agriculture Experiment Station, published with approval of the director.

LITERATURE CITED

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. 1987–1994. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

Aviv, H., and Leder, P. 1972. Purification of active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA 69:1408-1412.

Berger, S. L., and Chirgwin, J. M. 1989. Isolation of RNA. Methods Enzymol. 180:3-13.

Blobel, G., and Dobberstein, B. 1975. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. 67:835-851.

Bullock, W. O., Fernandez, J. M., and Short, J. M. 1987. XL1-Blue: A high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. Biotechniques 5:376-379.

Byrd, A. D., Schardl, C. L., Songlin, P. J., Mogen, K. L., and Siegel, M. R. 1990. The β–tubulin gene of *Epichloë typhina* from perennial ryegrass (*Lolium perenne*). Curr. Genet. 18:347-354.

Choi, G. H., Marek, E. T., Schardl, C. L., Richey, M. G., Chang, S., and Smith, D. A. 1990. *sti35*, a stress-responsive gene in *Fusarium* spp. J. Bacteriol. 172:4522-4528.

Choi, G. H., Richey, M. G., Turbek, C. S. M., and Smith, D. A. 1987. Comparative characteristics of a kievitone hydratase-producing isolate of *Fusarium solani* f. sp. *phaseoli* and of two strains deficient in this enzyme. Phys. Mol. Plant Pathol. 30:215-224.

Cleveland, T. E., and Smith, D. A. 1983. Partial purification, and further characterization, of kievitone hydratase from cell-free culture filtrates of *Fusarium solani* f. sp. *phaseoli*. Physiol. Plant Pathol. 22:129-142.

Dixon, R. A. 1986. The phytoalexin response: Elicitation, signalling and control of host gene expression. Biol. Rev. 61:239-291.

Gebeyechu, G., Rao, P. Y., SooChan, P., Simms, D. A., and Levan, L. 1987. Novel biotinylated nucleotide analogs for labeling and colorimetric detection of DNA. Nucleic Acids Res. 15:4513-4534.

Glass, N. L., and Kuldau, G. A. 1992. Mating type and vegetative incompatibility in filamentous ascomycetes. Annu. Rev. Phytopathol. 30:201-224.

Griffin, L. D., MacGregor, G. R., Muzny, D. M., Harter, J., Cook, R. G., and McCabe, E. R. B. 1989. Synthesis and characterization of a bovine hexokinase 1 cDNA probe by mixed oligonucleotide primed amplification of cDNA using high complexity primer mixtures. Biochem. Med. Metab. Biol. 41:125-131.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 1670:557-580.

Horton, R. M., Cai, Z., Ho, S. N., and Pease, L. R. 1990. Gene splicing by overlap extension: Tailor-made genes using the polymerase chain reaction. Biotechniques 8:528-535.

Kozak, M. 1989. The scanning model for translation: An update. J. Cell Biol. 108:229-241.

Kuhn, P. J., and Smith, D. A. 1979. Isolation from *Fusarium solani* f. sp. *phaseoli* of an enzymic system responsible for kievitone and phaseollidin detoxification. Physiol. Plant Pathol. 14:179-190.

Loh, E. Y., Elliott, J. F., Cwirla, S., Lanier, L. L., and Davis, M. M. 1989. Polymerase chain reaction with single-sided specificity: Analysis of T cell receptor δ chain. Science 243:217-220.

Lucy, M. C., Matthews, P. S., and VanEtten, H. D. 1988. Metabolic detoxification of the phytoalexins maackiain and medicarpin by *Nectria haematococca* field isolates: Relationship to virulence on chickpea. Phys. Mol. Plant Pathol. 33:187-199.

Mackintosh, S. F., Matthews, D. E., and VanEtten, H. D. 1989. Two additional genes for pisatin demethylation and their relationship to the pathogenicity of *Nectria haematococca* on pea. Mol. Plant-Microbe Interact. 2:354-362.

Marchuk, D., Drumm, M., Saulino, A., and Collins, F. S. 1991. Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. 19:1154.

Miao, V. P., Covert, S. F., and VanEtten, H. D. 1991a. A fungal gene for antibiotic resistance on dispensable ("B") chromosome. Science 254:1773-1776.

Miao, V. P. W., Matthews, D. E., and VanEtten, H. D. 1991b. Identification and chromosomal locations of a family of cytochrome P-450 genes for pisatin detoxification in the fungus *Nectria haematococca*. Mol. Gen. Genet. 226:214-223.

Miao, V. P. W., and VanEtten, H. D. 1992. Genetic analysis of the role of phytoalexin detoxification in virulence of the fungus *Nectria haematococca* on chickpea (*Cicer arietinum*). Appl. Environ. Microbiol. 58:809-814.

Müller, K. O. 1956. Einige einfache Versuche zum Nachweis von Phytoalexinen. Phytopathologische 27:237-254.

Paxton, J. D. 1981. Phytoalexins—A working redefinition. Phytopathol. Z. 101:106-109.

Reimmann, C., and VanEtten, H. D. 1994. Cloning and characterization of the *PDA6-1* gene encoding a fungal cytochrome P-450 which detoxifies the phytoalexin pisatin from garden pea. Gene 146:221-226.

Roy, K. W., Lawrence, G. W., Hodges, H. H., McLean, K. S., and Killebrew, J. F. 1989. Sudden death syndrome of soybean: *Fusarium solani* as incitant and relation of *Heterodera glycines* to disease severity. Phytopathology 79:191-197.

Rupe, J. C. 1989. Frequency and pathogenicity of *Fusarium solani* recovered from soybeans with sudden death syndrome. Plant Dis. 73:581-585.

Schäfer, W., Straney, D., Ciuffetti, L., Van Etten, H. D., and Yoder, O. C. 1989. One enzyme makes a fungal pathogen, but not a saprophyte, virulent on a new host plant. Science 246:247-249.

Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. 1988. λZAP: A bacteriophage λ expression vector with *in vivo* excision properties. Nucleic Acids Res. 16:7583-7599.

Shyamala, V., and Ames, G. F. L. 1989. Genome walking by single-specific-primer polymerase chain reaction: SSP-PCR. Gene 84:1-8.

Smith, D. A., and Banks, S. W. 1986. Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. Phytochemistry 25:976-995.

Smith, D. A., Harrer, J. M., and Cleveland, T. E. 1981. Simultaneous detoxification of phytoalexins by *Fusarium solani* f. sp. *phaseoli*. Phytopathology 71:1212-1215.

Smith, D. A., Harrer, J. M., and Cleveland, T. E. 1982. Relation between production of extracellular kievitone hydratase by isolates of *Fusarium* and their pathogenicity on *Phaseolus vulgaris*. Phytopathology 72:1319-1323.

Stahl, E. 1965. Thin-Layer Chromatography—A Laboratory Handbook. Springer-Verlag, New York.

Tegtmeier, K. J., and VanEtten, H. D. 1982. The role of pisatin tolerance and degradation in the virulence of *Nectria haematococca* on peas: A genetic analysis. Phytopathology 72:608-612.

Toneguzzo, F., Glynn, S., Levi, E., Mholsness, S., and Hayday, A. 1988. Use of a chemically modified T7 DNA polymerase for manual and automated sequencing of supercoiled DNA. Biotechniques 6:460-469.

Tsai, H.-F., Siegel, M. R., and Schardl, C. L. 1992. Transformation of *Acremonium coenophialum*, a protective fungal symbiont of the grass *Festuca arundinacea*. Curr. Genet. 22:399-406.

Turbek, C. S., Li, D., Choi, G. H., Schardl, C. L., and Smith, D. A. 1990. Induction and purification of kievitone hydratase from *Fusarium solani* f. sp. *phaseoli*. Phytochemistry 29:2841-2846.

Turbek, C. S., Smith, D. A., and Schardl, C. L. 1992. An extracellular enzyme from *Fusarium solani* f. sp. *phaseoli* which catalyzes hydration of the isoflavonoid phytoalexin, phaseollidin. FEMS Microbiol. Lett. 94:187-190.

VanEtten, H. D., Matthews, D. E., and Matthews, P. S. 1989. Phytoalexin detoxification: Importance for pathogenicity and practical implications. Annu. Rev. Phytopathol. 27:143-164.

Vieira, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.

Vogelstein, B., and Gillespie, D. 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA 76:615-618.

Vollmer, S. J., and Yanofsky, C. 1986. Efficient cloning of genes in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 83:4869-4873.

von Heijne, G. 1986. Towards a comparative anatomy of N-terminal topogenic protein sequences. J. Mol. Biol. 189:239-242.

Wang, W. P., Myers, R. L., and Chiu, I. M. 1991. Single primer-mediated polymerase chain reaction: Application in cloning of two different 5′-untranslated sequences of acidic fibroblast growth factor messenger RNA. DNA Cell Biol. 10:771-777.

Weltring, K.-M., Turgeon, B. G., Yoder, O. C., and VanEtten, H. D. 1988. Isolation of a phytoalexin-detoxification gene from the plant pathogenic fungus *Nectria haematococca* by detecting its expression in *Aspergillus nidulans*. Gene 68:335-344.

Yelton, M. M., Hamer, J. E., and Timberlake, W. E. 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc. Natl. Acad. Sci. USA 81:1470-1474.

Yelton, M. M., Timberlake, W. E., and van den Hondel, C. A. M. J. J. 1985. A cosmid for selecting genes by complementation in *Aspergillus nidulans*: Selection of the developmentally regulated *yA* locus. Proc. Natl. Acad. Sci. USA 82:834-838.

Zhang, Y., and Smith, D. A. 1983. Concurrent metabolism of the phytoalexins phaseollin, kievitone and phaseollinisoflavan by *Fusarium solani* f. sp. *phaseoli*. Physiol. Plant Pathol. 23:89-100.