Association of a Virus with Wheat Displaying Yellow Head Disease Symptoms in the Great Plains

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ABSTRACT

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Wheat with yellow head disease (YHD) (yellow heads and mosaic leaf symptoms) has been observed in Kansas since 1997. A pathogen was transmitted from the infected wheat to maize by vascular puncture inoculation and to *Nicotiana benthamiana* by rub inoculation. The original infected wheat and infected maize and *N. benthamiana* test plants all produced a unique 32- to 34-kDa protein when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Time-of-flight mass spectrometry analysis of the unique 32- to 34-kDa protein showed that the amino acid sequence was most closely related to the nucleoprotein of *Rice hoja blanca virus*, indicating that the virus causing YHD symptoms in wheat is a tenuivirus. Antiserum made to this protein failed to react with extracts made from healthy wheat or wheat infected with *Wheat streak mosaic virus* or the High Plains virus. The antiserum did react to extracts made from symptomatic wheat, maize, and *N. benthamiana*, shown by SDS-PAGE to contain the unique protein, and to extracts of wheat with YHD symptoms from Kansas, North Dakota, South Dakota, and Oklahoma. The name Wheat yellow head virus is proposed for this virus.

Wheat (*Triticum aestivum* L.) is an important crop grown throughout the Great Plains region of the United States. In Kansas and surrounding states, it is infected naturally by *Agropyron mosaic virus* (AgMV) (27), *Wheat American striate mosaic virus* (WASMV) (29), *Barley yellow dwarf mosaic virus* (25), High Plains virus (HPV) (16), *Wheat soilborne mosaic virus* (5), *Wheat spindle streak mosaic virus* (41), and *Wheat streak mosaic virus* (WSMV) (40).

Viruses in the genus *Tenuivirus* include *Echinochloa hoja blanca virus* (EHBV), *Maize stripe virus*, *Rice grassy stunt virus*, *Rice stripe virus*, *Urochloa hoja blanca*

virus (UHBV), and *Rice hoja blanca virus* (RHBV), with each virus species being vectored by a different species of planthopper (23). In the United States, RHBV has been reported infecting rice in Florida in 1957 (1), in Mississippi in 1958 (2), and in Louisiana in 1960 (3). The vector of RHBV (*Tagosodes orizicolus*) (23) also was identified in Mississippi (2) and Louisiana (3). However, RHBV has not been reported to infect plants in the Great Plains, nor has its vector been identified in this region.

In 1997, wheat plants with yellow heads and mosaic symptoms on the flag leaf were observed in the field; however, tests by enzyme-linked immunosorbent assay (ELISA) failed to identify any of the viruses previously found infecting wheat in Kansas. However, using vascular puncture inoculation (VPI) of embryonic tissue of maize seed, an infectious agent was transmitted to maize test plants.

We present evidence that wheat plants with yellow head symptoms are associated with a virus and that it is most closely related to RHBV and propose the name Wheat yellow head virus (WYHV).

MATERIALS AND METHODS

Virus source, maintenance, and field sampling. In May 1997, 30 wheat plants from plots of Custer and Denominator

wheat (six rows of wheat, 0.3 m apart by 17 m) were selected at Hays, KS that had yellow heads and mosaic symptoms on only the flag leaf, except for one plant which also had mosaic symptoms on the penultimate leaf. Ten plants were randomly selected again in 1998 and 2000 at Hays.

The WYHV-KS97 strain (from wheat collected in May 1997) was propagated in maize ('Spirit') using VPI (22,32). Briefly, maize seed were inoculated by placing WYHV inoculum on the embryo side of each kernel, after which the kernel was inoculated with five minuten pins affixed to an engraver through the inoculum and the pericarp covering of the scutellum. The seed were incubated at 30°C in a moist environment for 48 h and then planted into soil. VPI was conducted during the investigations using, as inoculum sources, Kansas wheat with yellow head symptoms isolated in 1997 (WYHV-KS97) and 2000 (KSU00-105), as well as one wheat plant with yellow head symptoms from North Dakota (NDV).

Positive RHBV controls were in rice (lyophilized) obtained from Francisco Morales (CIAT, Cali, Colombia). Positive controls consisted of frozen or fresh tissue of the Sidney 81 isolate (42), frozen maize tissue infected with HPV (33), frozen wheat tissue infected with AgMV (27), and frozen wheat tissue infected with WASMV (29).

Virus purification, antiserum preparation, and antiserum sources. WYHV-KS97 was purified from maize infected using VPI for production of antibodies as described previously (27). Briefly, WYHV-infected maize was ground in grinding buffer (GB; 0.1 M diammonium citrate, 0.25% [vol/vol] 2-mercaptoethanol, 0.1% [vol/vol] sodium diethyldithiocarbamate, and 1% polyvinylpyrrolidone, MW 40,000) at a ratio of 1 g of tissue per 7 ml of GB, filtered through cheesecloth, and adjusted to a concentration of 4% (vol/vol) Triton X-100. The plant extract was centrifuged at 23,700 × g for 15 min at 5°C. The supernatant was pipetted into 50 Ti (Beckman Coulter, Fullerton, CA) centrifuge tubes and underlaid with 2 ml of 0.988 M sucrose in GB and centrifuged for

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2 h at $85,000 \times g$ at 5°C . The supernatant was discarded and the pellets were resuspended in 3.5 ml of GB. The resuspended pellets then were centrifuged for 10 min at $31,000 \times g$. The extract then was transferred to SW 50.1 centrifuge tubes (Beckman Coulter) and underlaid with 1.0 ml of 2.33 M CsCl and 0.5 ml of 3.297 M CsCl. The preparations then were centrifuged for 3 h at $139,000 \times g$ at 5°C . After centrifugation, a syringe with an attached bent needle was used to collect the banded virus. The collected virus was made to 5 ml with GB and pelleted by centrifugation for 1 h at $85,000 \times g$ at 5°C .

A 6-week-old New Zealand white female rabbit was injected intramuscularly in the hind legs with approximately 100 μg of purified WYHV from different preparations that were each dissolved in 1.0 ml of phosphate buffered saline (PBS), pH 7.0. The solution then was emulsified with an equal volume of Freund's incomplete adjuvant. Injections were given four times on a weekly basis. Bleeding for the collection of antibodies was started after 3 weeks and carried on once a week for 4 weeks.

Antiserum to the following viruses was prepared or obtained as described previously, and dilutions were made from stocks of 1 mg/ml: WSMV (1:1,000 vol/vol) (27), HPV (1:50,000 vol/vol) (16,33), WASMV (1:600 vol/vol) (29), and AgMV (1:1,000 vol/vol) (27). The RHBV antiserum to the virus-specific major non-capsid protein (MNP) (1:1,600 vol/vol) (10) was obtained from Bryce Falk (University of California, Davis) and that to the RHBV nucleoprotein (N-protein) (1:1,600 vol/vol) from Francisco Morales (CIAT, Cali, Colombia).

Indirect ELISA. Indirect ELISA was as described previously (34). Leaf tissue was ground 1:30 (wt/vol) in 0.05 M carbonate buffer, pH 9.6 (coating buffer) (6), and antiserum to WYHV was used at a 1:800 (vol/vol) dilution. The anti-rabbit antibody alkaline phosphatase conjugate (1:3,000 vol/vol) (Sigma-Aldrich, St. Louis) was diluted using ELISA blocking buffer (5% nonfat dry milk, 0.01% antifoam A, 0.02% sodium azide, in PBS, pH 7.4). All samples, antibody solutions, and substrate were used at a 200 μl per well. Substrate (p-nitrophenyl phosphate, 0.714 mg/ml) in substrate buffer (6) was added to the wells of the plates, incubated at room temperature, and the absorbance (405 nm) measured after 30 min (Titertek Multiskan Microelisa Plate Reader; Flow Laboratories, Inc., McLean VA). The minimum positive absorbance threshold value for all antisera was arbitrarily set at twice the healthy control.

Minipurification, sodium dodecyl sulfate gel electrophoresis, and Western blot analyses. Proteins were extracted and minipurified as described previously (31). Briefly, 1 g of WYHV-infected tissue was ground 1:7 (wt/vol) with a mortar and

pestle in 0.1 M ammonium citrate buffer, pH 6.5, containing 0.25% mercaptoethanol, 0.1% sodium diethyldithiocarbamate, and 1% polyvinylpyrrolidone. The extract was filtered through cheesecloth and centrifuged for 10 min at $5,900 \times g$. Triton X-100 then was added to the supernatant to a final concentration of 7.4% and centrifuged through a 1-cm layer of 0.988 M sucrose in ammonium citrate buffer for 2 h at $85,000 \times g$. The pellet was resuspended in 200 μl of sodium dodecyl sulfate (SDS) treatment buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol), heated for 4 min at 100°C , reduced, and alkylated (20); then, 25 μl was electrophoresed in 10% polyacrylamide gels. Gels were stained with Coomassie Blue R-250. Molecular weight markers used in Figure 1 were Sigma Markers (Sigma-Aldrich). Relative molecular mass was calculated using a densitometer to determine electrophoretic mobility (29) compared with protein standards (ProSieve protein markers; Bio-Products, Rockland, ME). SDS-polyacrylamide gel electrophoresis (PAGE) (18) analyses were carried out using electrophoresis and gel staining conditions described previously (30). Analyses were conducted three times using extracts from different symptomatic plants. Healthy tissue was treated and tested in an identical manner.

Western blotting, plant extractions, separations by SDS-PAGE, and transfer of proteins onto nitrocellulose membranes were done as described previously (30). Alkaline phosphatase goat anti-rabbit antibody conjugate (1:3,000 dilution) (Fisher Scientific, Denver, CO) was used for immunoblotting (4).

Protein amino acid sequence analysis by time-of-flight mass spectrometry. The WYHV protein unique to infected maize separated by SDS-PAGE was excised from the gel and sent to the University of Manitoba for time-of-flight mass spectrometry (TOFMS) analysis. Excised proteins were digested in-gel with sequencing-grade endoproteinases (Roche Diagnostic Corp., Indianapolis, IN) as described previously (37). Proteolytic digestions were carried out using various enzymes: by 50 ng of trypsin, Lys-C, Glu-C, or chymotrypsin in 25 mM ammonium carbonate; by Asp-N in 10 mM Tris-HCl; or by pepsin in 5% acetic acid (pH 2.5). Following digestion, peptide mass mapping and tandem mass spectrometry (MS/MS) measurements on the digests were carried out on a Manitoba/Sciex (MDS Sciex, Concord ON, Canada) prototype MALDI-QqTOF mass spectrometer (21), using 2,5-dihydroxybenzoic acid as the matrix.

Chemical derivatization of the digests by sulfonic acid at the N-terminus was used in some cases to aid peptide sequence identification (17,37). Alternatively, de novo peptide sequencing was carried out

by O^{18} labeling of the C-terminal tryptic ions (39). In the latter, the b and y series ions were distinguished by blotting the gel-separated proteins onto a nitrocellulose membrane followed by in-solution tryptic digestion in 10 μl of O^{18} water (38).

The protein database was first searched by MS-Tag. If no hit was obtained, the observed MS/MS data were analyzed by manual interpretation, followed by a BLAST or FASTA homology database search.

WYHV distribution. In May 2001, wheat samples with WYHV symptoms collected from random locations in South Dakota were sent to our lab by Marie Langham (South Dakota State University, Brookings). In May 2002, wheat samples collected from Lahoma and Burlington in Oklahoma were sent by Bob Hunger (Oklahoma State University, Stillwater), and in July by Marcia McMullen (North Dakota State University, Fargo) from spring wheat at Tulna and Fargo, ND. The 2001 samples were tested against WYHV antiserum only, whereas the 2002 Oklahoma and North Dakota samples were tested against WYHV antiserum and also against RHBV N-protein antiserum that was not available in 2001. In addition, all samples were tested against antiserum to WSMV and HPV.

Host range. Plants used in host range evaluations included 'Spirit' maize (*Zea mays* L.), 'Tomahawk' wheat (*Triticum aestivum* L.), 'Westford' barley (*Hordeum vulgare* L.), 'GATE 76' sorghum (*Sorghum bicolor* L. Moench), 'Bonel' rye (*Secale cereale* L.), 'Lodi' oat (*Avena sativa* L.), and *Nicotiana benthamiana*. Except for *N. benthamiana*, seed were planted into 30-by-50-cm soil-filled metal flats in a greenhouse. Seedlings were inoculated (1:10 wt/vol) mechanically (26) at the two-to-three-leaf stage with extracts from wheat plants systemically infected with WYHV isolates from Kansas collected in 1997 and 2000. *N. benthamiana* seed were planted in pine cells (Stuewe and Sons, Inc. Corvallis, OR) and thinned to one plant per cell. The first two leaves were inoculated at the two-leaf stage by dipping a Q-tip into plant extract containing 1% Crystolon flour B and 600-mesh carborundum (Norton Co., Worcester, MA) and gently rubbing the adaxial leaf surfaces. All seed for plants used in host range experiments were obtained from sources at the Kansas State University Agricultural Research Center at Hays. Experiments were conducted twice with 6 to 10 plants per species used and 25 plants of *N. benthamiana*.

Vector experiments. In 1999, transmission tests were conducted with six aphid species: Greenbug (*Schizaphis graminum* Rondani), Russian wheat aphid (*Duraphis noxia* Mordvilko), corn leaf aphid (*Rhopalosiphum maidis* (Fitch)), bird cherry-oat aphid (*R. padi* (L.)), green peach aphid (*Myzus persicae* (Sulzer)), and the English

grain aphid (*Macrosiphum avena* (F.)). Aphids were held on WYHV-infected Spirit maize plants using cages designed for retention of wheat curl mites (33) for 24 h, and then individually transferred directly to healthy Tomahawk wheat seedlings at the one- to two-leaf stage. Ten wingless adults and nymphs were confined in similar cages on each assay plant for 3 days, after which the cages and aphids were removed and the plants observed for symptoms for 3 weeks. Ten assay plants were infested with each aphid species and the test was repeated three times.

In 2000, tests were conducted with leafhopper species, including painted leafhopper (*Endria inimica* (Say)), gray lawn leafhopper (*Exiitatus exitiosus* (Uhler)), *Draeculacephala mollipes* (Say), *Macrosiotes* spp., and *Deltocephalus* spp. (identification by T. Harvey). All leafhoppers were collected by sweep net from wheat and native grasses during May, June, and

July. Leafhoppers collected in the field were transferred from the sweep net using an aspirator. Transfers were done at 5 to 6°C to reduce escapes while sorting and transferring the leafhoppers. After being confined in wheat curl mite cages (33) on infected seedlings of Spirit maize for 5 or more days, leafhoppers were transferred to and caged on healthy Tomahawk wheat at the one- to two-leaf stage. Each of 10 assay plants was infested with 10 leafhoppers of a given species. Leafhoppers were held on the assay plants for 3 weeks or until the leafhoppers died. After the inoculation period, the cages were removed and the plants were observed for symptoms.

RESULTS

Field sampling. All 30 plants sampled from the field at Hays, KS in May 1997 tested negative in ELISA for AgMV, HPV, WSMV, and WASMV. The estimated percentages of wheat plants with these symptoms were 1 and 5% for the Custer and Denominator plots, respectively. Tissue from plants 7, 8, 15, 16, 21, and 23 (all negative in ELISA) was bulked (coded as WYHV-KS97) and used in a minipurification experiment. The tissue showed a unique band that measured 33 kDa in SDS-PAGE that was not present in extracts from healthy wheat (*data not shown*).

Vascular puncture inoculation. SDS-PAGE gels showed a unique band associated with symptomatic maize (infected using wheat as the pathogen source) from both the minipurified and CsCl preparations (Fig. 1). The protein was not present in extracts from healthy maize preparations. Symptomatic maize from the first transmission by VPI was used to inoculate Spirit maize seed, resulting in 33 of 87 plants with similar mosaic symptoms. Plants from this experiment were negative in ELISA against WSMV and HPV antiserum; however, the 32- to 34-kDa protein again was observed on SDS-PAGE gels when bulked leaf tissue was subjected to both minipurification and CsCl purification (Fig. 1). A third transmission cycle using symptomatic leaf tissue from several of the 33 symptomatic maize plants as inoculum for VPI of Spirit maize seed resulted in 12 of 90 maize plants developing systemic symptoms. Similar results

were obtained with extracts from bulked leaf tissue (Fig. 1).

WYHV antiserum. At each antiserum dilution, samples taken at 49 days post inoculation (dpi) resulted in the highest ELISA value, followed by the 28- and 26-dpi samples (Table 1). The titration end-point was 1:1,600 at this dilution, and ELISA absorbance values were below 0.100. A fivefold maximum increase in ELISA values for WYHV-infected tissue occurred at each antiserum dilution when compared with the healthy controls. The antiserum did not react to WSMV or HPV (*data now shown*).

In 1998, all seven yellow wheat heads tested positive against the antiserum, as did leaves with mosaic symptoms (Table 2). Numbers of symptomatic and ELISA-positive leaves decreased progressively below the flag leaf, with only one plant having a symptomatic F-2 leaf. All symptomless heads and leaf tissue from plants in this test were negative in ELISA. In 2000, only symptomatic heads and leaves were positive in ELISA. No plant had a symptomatic F-2 leaf and only one plant had a symptomatic F-1 leaf (ELISA value of 0.086). Consistent with the 1998 sampling, all symptomless tissues from 2000 were negative in ELISA against WSMV and HPV antiserum.

TOFMS. Analysis of the amino acid sequence of the 32- to 34-kDa protein unique to WYHV-infected wheat and maize plants by TOFMS showed that it was most closely related to the N-protein of the tenuiviruses RHBV (AAB69772), followed by UHBV (AAB58305), and EHBV (AAB07227) (Fig. 2). The N-terminus of WYHV-specific protein was acetylated. Its sequence consisted of 314 amino acids (aa) compared with 319 aa for RHBV, with the amino acid deletions in WYHV corresponding to positions 5, 254, 256, 258, and 259 of the RHBV counterpart sequence. Of the 314 aa in the WYHV sequence, 102 were different from that of RHBV, resulting in a 68% identity. In all, 5 of 9 aa residues at the N-terminal and 6 of 9 aa residues in the C-terminal region were substitutions of their RHBV counterparts. The WYHV-specific protein had a molecular weight of 35,373 Da calculated from the sum of its 314 aa. The Wheat yellow head virus N-protein sequence has been

Fig. 1. Protein concentrated from 'Spirit' maize (infected and healthy) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane 1, molecular weight markers; lane 2, protein pellet from minipurification phase of symptomatic maize; lane 3, band from CsCl separation of symptomatic maize taken 8.0 to 8.5 mm from the bottom of centrifuge tube; lane 4, band from CsCl separation of symptomatic maize taken 9.5 to 10 mm from bottom of centrifuge tube; lane 5, protein pellet from minipurification phase of healthy maize; lane 6, zone removed from CsCl separation of healthy maize taken 8.0 to 8.5 mm from bottom of centrifuge tube; lane 7, zone removed from CsCl separation of healthy maize taken 9.5 to 10 mm from bottom of centrifuge tube.

Table 1. Enzyme-linked immunosorbent assay (ELISA) values for dilutions of the 'Spirit' maize infected with a 1997 isolate of Wheat yellow head virus (WYHV) at different days post inoculation (DPI) and for the healthy maize (HM) control

Sample	DPI	Antiserum dilutions ^a									
		1:100		1:200		1:400		1:800		1:1,600	
		ELISA	GHV	ELISA	GHV	ELISA	GHV	ELISA	GHV	ELISA	GHV
WYHV	49	0.406	5.1	0.277	5.6	0.192	5.2	0.122	5.3	0.080	5.3
WYHV	28	0.297	3.7	0.192	3.9	0.126	3.4	0.088	3.8	0.053	3.5
WYHV	26	0.255	3.2	0.160	3.2	0.108	2.9	0.065	2.8	0.042	2.8
HM	...	0.079	1.0	0.049	1.0	0.037	1.0	0.023	1.0	0.015	1.0

^a Antiserum dilutions prepared from a stock solution of whole antiserum at 1 mg/ml. GHV = Number of times greater (calculated by dividing the ELISA value by that of the healthy control, which is shown as 1.0) than the healthy control. Values presented are representative of two additional experiments.

deposited as Swiss Prot. accession no. P84254.

Serological relationship of WYHV. Proteins from extracts of wheat and maize infected by the WYHV-KS97 isolate and wheat and *N. benthamiana* infected by the NDV all showed a unique protein band migrating to a similar position (32 to 34 kDa) when separated by SDS-PAGE (Fig. 3A). A protein band from RHBV-infected rice also migrated to approximately the same position. The proteins were not observed in extracts from healthy maize, wheat, *N. benthamiana*, or rice. Results of Western blots showed that only the Kansas isolate of WYHV-KS97 and the NDV protein band reacted with the WYHV antibody probe (Fig. 3B). In an equivalent Western blot probed with antiserum to the N-protein of RHBV, WYHV, NDV, and the RHBV-positive control, all reacted positively (Fig. 3C). When probed with the antiserum to RHBV MNP, only Kansas WYHV-KS97 in wheat, NDV in wheat, and *N. benthamiana* and RHBV in rice reacted positively (Fig. 3D). Faint reaction bands were observed from 27.8 to 35 kDa using the MNP noncapsid protein probe (Fig. 3D), and a single band occurred at approximately 49.6 kDa using the anti-RHBV capsid protein probe (Fig. 3C). The data presented are representative of three experiments.

WYHV antiserum reacted strongly to tissue infected by either the Kansas WYHV or the NDV isolates, but reacted only weakly to the RHBV control (Table 3). The antiserum made to the RHBV N-protein reacted weakly to the WYHV-KS97- and NDV-infected tissue and robustly to the RHBV-positive control. The antiserum reacted with healthy rice as well as wheat infected with WYHV or NDV. Because the Kansas and North Dakota samples were wheat extracts, healthy wheat value was used to calculate the greater-than-healthy control values as indicated in Table 3. The RHBV nonstructural antiserum reacted robustly to both the Kansas WYHV and NDV, less so to the RHBV-positive control, and had little or no reaction to healthy wheat or rice. The data presented are representative of three experiments.

Virus distribution. The 11 heads of wheat with YHD symptoms from South Dakota all were positive in ELISA, with an average absorbance value of 0.120 (range 0.056 to 0.208) against WYHV antiserum (Table 4). Of the 11 wheat samples, 6 had flag leaves with mosaic symptoms that were positive in ELISA, with an average value of 0.201 (range 0.110 to 0.276). The remaining five symptomless leaves resulted in an average value of 0.024 (range 0.012 to 0.033). Because the minimum threshold for a positive ELISA was arbitrarily set at twice the healthy control, some of the ELISA values for the symptomless leaves fell within the positive

Table 2. Average enzyme-linked immunosorbent assay (ELISA) values against Wheat yellow head virus (WYHV) antiserum from either symptomatic (S) or nonsymptomatic (NS) heads (H), flag leaves (F), the leaf subtending the flag leaf (F-1) and the second leaf below the flag leaf (F-2) of naturally infected field-grown wheat collected in 1998 and 2000 at Hays, KS^a

Sample type	Year of field sampling ^b			
	1998		2000	
	S	NS	S	NS
H	0.170 (7)	0.020 (3)	0.208 (10)	NV (0)
F	0.174 (6)	0.018 (4)	0.195 (8)	0.060 (2)
F-1	0.152 (2)	0.023 (8)	0.086 (1)	0.035 (9)
F-2	0.080 (1)	0.015 (9)	NV	0.041 (10)
Healthy wheat H	...	0.018	...	0.049
Healthy wheat F	...	0.020	...	0.033
WYHV control ^c	0.161		0.133	

^a Ten plants were used each year of sampling.

^b Number in parenthesis following each ELISA value indicates the number of plants whose ELISA values were used to calculate the average ELISA value; NV = no ELISA value because tissue with that symptom type was not present in any of the 10 plants tested.

^c WYHV was frozen leaf tissue from the original 1997 isolate in 'Spirit' maize from which the antiserum was prepared.

1	UHBV	MAMSVADVQT	EIERVTALAL	KYITDHKDTL	VTFTVGQIEYN	GYDAGKLLQI	50
	RHBV	MTMSVADVQT	EIERVTTLAL	KYITDHKDTL	VTFTVGQIEYN	GYDAGKLLQI	
	EBHV	. .MSVADIQT	EIERVTALAL	KYIADHKDTL	VTFTVGQIEYN	GYDAGKLLQI	
	WYHV	@--SM-.EI-N	-----A--V	---SEN--S-	-V-----D--	-----S-	
51	UHBV	LKKKAEGRDF	GKDLCYLLVM	RYTRGTGFVR	DVRKKIKTAA	GGDTAHEIVT	100
	RHBV	LKKKAEGRDF	GKDLCYLLVM	RYTRGTGFVR	DVRKKIKTAA	GGDTAHEIVT	
	EBHV	LKKKAEGRDF	GKDLGFLLVV	RYTRGTGFVR	DVRKKIKTAA	GGDTAHEIVT	
	WYHV	--E--K----	-R--CY---M	-----	-----V--	-A---Y----	
101	UHBV	HYGVVHSVGD	NADAITLGRL	ASLFFAVSMN	IVRNVSTGAK	LAVDSSDLGS	150
	RHBV	HYGVVHSVGD	NADAITLGRL	ASLFFAVSMN	IVRNVSTGAK	LAVDSSDLGS	
	EBHV	HYGVVHSVGD	NADAITLGRL	ASLFFAVSMS	IVRNVSSGAK	LAVDSSDLGS	
	WYHV	-----	-----	-A---Y---N	--KS--T---	--L-T----T	
151	UHBV	SGLDLLLWDF	VPQFITLDSV	TAPYCSGKGM	NIILFSLHVL	HGALTTKKTM	200
	RHBV	SGLDLLLWDF	VPQFITLDSL	TAPYCTGKGM	NTILFSLHVL	HGALTTKKTM	
	EBHV	SGLDLLLWDF	VPQFITLDSV	TAPYCTGKGM	NVILFSLHVL	HGALTTRKTM	
	WYHV	-----	-----N---V	D---NK-NT	SN-----L-	Q-----R---	
201	UHBV	PDQKKKARNL	VTDFELVKYT	TELLVITCNA	KNLKAEEKTS	YRESLMRPFQ	250
	RHBV	PDQKKKARSL	VTDFELVKYT	TELLVITCNA	KNLKVKKTI	YRESLMRPFQ	
	EBHV	PDLKKKARSL	VTDFELVKYT	TELLVITCNA	KNLKAERKTA	YRKSLSMSFQ	
	WYHV	--Q---KDN-	T---D-L---	A-----S-	---TDNKKST	--KK-VEP-R	
251	UHBV	EGAGDALGFK	ETFWTSLAKV	SNGCVRKMCK	DAQTYLSERT	SLLKVFVDNC	300
	RHBV	EGAGDALGFK	ETFWTSLAKV	STGCVRKMCK	DAQAYLSERT	PLKVFVENC	
	EBHV	EGAGDDLGFK	ESFWTSLAKV	STGCVRKMCK	DAQAYLSERT	SLLKLFVENC	
	WYHV	-NE.-.Y.-.	AD---A-G-L	-T--LK----	---N--KD--	TV--LM-D--	
301	UHBV	NKAEAEVTEA	IKAYFVTES				319
	RHBV	NKSEDEVAEA	IKSYFVTGA				
	EBHV	NKTEAEVTEA	IKSYFVTDA				
	WYHV	SGTDD-AAK-	--D-LTVDD				

Fig. 2. Amino acid sequence of the protein unique to wheat and maize infected by Wheat yellow head virus (WYHV) as determined by time-of-flight mass spectrometry. The WYHV sequence is compared with *Urochloa hoja blanca* virus (UHBV-AAB58305), *Rice hoja blanca* virus (RHBV-AAB69772), and *Echinochloa hoja blanca* virus (EBHV-AAB07227). The symbol @ refers to N-terminal acetylation, dashed lines in the WYHV sequences indicate consensus with RHBV, and a dot (.) indicates a deletion of an amino acid at that position.

threshold. All plants were negative for WSMV and HPV in ELISA. The South Dakota samples were not tested against RHBV antiserum because it was not available in 2001.

All heads from the 15 Oklahoma wheat samples were symptomatic and all tested positive in assays using WYHV as well as RHBV antiserum, resulting in average values of 0.192 (range 0.105 to 0.291) and 0.368 (range 0.283 to 0.483), respectively (Table 4). Five of the Oklahoma plants also were positive against WSMV antiserum. For the 15 North Dakota wheat samples, all heads were yellow and all but three flag leaves were rated as having mosaic symptoms. However, all heads were positive against WYHV and RHBV antiserum, resulting in average ELISA values of 0.163

(range 0.105 to 0.243) and 0.205 (range 0.142 to 0.265), respectively. The 12 symptomatic flag leaves all tested positive for WYHV, with average values of 0.256 (range 0.096 to 0.434). For the North Dakota RHBV sampling, 9 of the 12 symptomatic flag leaves tested positive, with an average value of 0.142 (range 0.061 to 0.216). However, 3 of the 12 symptomatic flag leaves reacted very weakly to the RHBV, resulting in values of 0.038, 0.045, and 0.021, while the same leaves tested strongly against the WYHV antiserum, with values of 0.434, 0.325, and 0.304, respectively. No RHBV-positive control was available for these tests because it was consumed in the Western blotting experiments (Fig. 3) and in ELISA tests determining serological reaction to RHBV (Table 3).

Host range and vector studies. Barley, wheat, sorghum, rye, maize, and oat plants mechanically inoculated with WYHV-KS97 did not develop symptoms, and extracts from bulked leaves of these plants did not contain the band unique to WYHV-KS97 in SDS-PAGE analysis in any of the three experiments. However, *N. benthamiana* mechanically inoculated with WYHV-KS-97 in the three experiments developed a very faint mosaic, and symptomatic plants were stunted (*data not shown*). The proportions of infected *N. benthamiana* test plants in each of the three experiments were 3/25, 2/25, and 5/25, respectively. When extracts from bulked leaves of symptomatic *N. benthamiana* (two leaves above the inoculated leaves) were

Fig. 3. A, Protein separated by minipurification and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis from maize, wheat, and *Nicotiana benthamiana* and probed with B, antibodies made to the nucleoprotein of Wheat yellow head virus-Kansas isolate (WYHV-KS97); C, antibodies to the nucleoprotein (N-protein) of *Rice hoja blanca virus* (RHBV); and D, antibodies to the major nonstructural proteins of RHBV. A, Lane 1, molecular mass standards; lanes 2 and 3, WYHV-KS97 in maize and wheat, respectively; lanes 4 and 5, North Dakota virus (NDV) in wheat and *N. benthamiana*, respectively; lane 6, RHBV in rice; lanes 7–10, healthy maize, wheat, *N. benthamiana*, and rice, respectively. Arrows in lanes 2 and 3 mark the location of the protein associated with WYHV, in lanes 4 and 5 the protein associated with the NDV, and in lane 6 the N-protein of RHBV. B, C, and D, Samples are the same as described for A, except that lane 1 contains prestained mass markers. Arrows or arrowheads mark the locations of proteins from WYHV-infected plants reacting to B, antibodies of WYHV, C, antibodies to the RHBV N-protein, and D, RHBV nonstructural antiserum probes.

tested by minipurification followed by SDS-PAGE, the band unique for WYHV-KS97 was present in the plants (*data not shown*).

N. benthamiana infected with WYHV-KS97 was used as a source for VPI and infected 17 of 83 Spirit maize plants. Bulk leaves from the maize infected using extracts from *N. benthamiana* used again to mechanically inoculate new healthy *N. benthamiana* resulted in 3 of 25 infected plants.

The experiment was repeated using *N. benthamiana* challenged with another Kansas isolate (KSU00-105) from a single wheat plant (Denominator) with yellow head symptoms that had tested negative in ELISA to WSMV and HPV. The extract from this sample infected 5 of 25 *N. benthamiana* plants, and symptoms resembled those induced by infection with the 1997 WYHV isolate. Extracts of *N. benthamiana* infected with KSU00-105 tested positive in ELISA only against WYHV antiserum, and minipurification produced a protein that migrated in SDS-PAGE to the

same position as that of the 1997 WYHV isolate (*data not shown*).

The North Dakota wheat samples that tested positive against WYHV antiserum were used to mechanically inoculate *N. benthamiana*, resulting in the infection of 4 of 25 *N. benthamiana* plants. The NDV in wheat and *N. benthamiana* both had a unique protein that migrated to a position in SDS-PAGE similar to that of the Kansas WYHV isolate, and reacted in Western blotting similarly to the Kansas WYHV isolate (Fig. 3).

In experiments with aphids in 1999 and leafhoppers in 2000, no wheat seedlings infested with these insects developed symptoms.

DISCUSSION

Wheat exhibiting YHD symptoms first was observed in 1997 and the disease has been detected every year since then, although the disease incidence has never exceeded 5%. We found that such symptomatic wheat had a unique protein band associated with it of approximately 32 to

34 kDa as estimated by SDS-PAGE, and that a pathogen could be transmitted to Spirit maize by VPI. The maize test plants developed mosaic symptoms and, when analyzed, had a similar band that co-migrated with the 32- to 34-kDa protein from wheat (Figs. 1 and 3). We could re-inoculate successive rounds of maize test plants with extract made from symptomatic maize, and each successive transfer produced plants with similar symptoms accompanied by the 32- to 34-kDa protein. We also were able to transmit the agent to *N. benthamiana* by rub inoculation in successive trials from wheat or maize, and re-infect maize or *N. benthamiana*. In these experiments, there was a consistent association with the presence of the 32- to 34-kDa protein in SDS-PAGE analyses (Fig. 3). Taken together, Koch's postulates were fulfilled based on successful transmission of the WYHV-KS97, KSU00-105, and NDV isolates to maize and *N. benthamiana* test plants. In wheat, the postulates were not fulfilled because the virus associated with the 32- to 34-kDa band was not mechanically transmissible by conventional leaf-rub inoculation or by VPI. The failure of VPI to transmit the WYHV isolates to wheat may be simply a mechanical phenomenon arising from wheat's smaller seed size and the attendant difficulty of performing VPI. We experienced similar difficulty in studies of HPV, in which we could transmit the disease agent to maize by VPI but not to wheat (33).

In initial stages of this investigation, the available evidence suggested that the WYH disease agent might be a variant of HPV because both possessed an N-protein of approximately 32 kDa (16,33). However, HPV is not known to cause yellow head symptoms (31,33). Antiserum made to CsCl-purified WYHV reacted in Western blot experiments specifically to the 32- to 34-kDa band (Fig. 3) and reacted only to extracts of wheat (Kansas, North Da-

Table 3. Enzyme-linked immunosorbent assay (ELISA) values against antisera to Wheat yellow head virus (WYHV) and *Rice hoja blanca virus* (RHBV) nucleocapsid (RHBV-NP) and major nonstructural protein (RHBV-MNP) from symptomatic leaf tissue of wheat infected with the 1997 Kansas isolate of WYHV (WYHV-KS) and from naturally infected wheat with yellow head symptoms from North Dakota (NDV) in 2002^a

Sample	Antisera ^b					
	WYHV	GHV	RHBV-NP	GHV	RHBV-MNP	GHV
WYHV-KS	0.427	14.7	0.303	6.9	0.575	144.0
NDV	0.327	11.3	0.234	5.2	0.641	160.2
RHBV ^c	0.072	2.5	1.086	24.7	0.328	92.0
Healthy rice	0.043	1.5	0.233	5.3	0.010	2.5
Healthy wheat	0.029	1.0	0.044	1.0	0.004	1.0

^a WYHV was from frozen wheat tissue from the original 1997 isolate and NDV = samples consisted of bulked symptomatic flag leaf tissue previously testing positive in preliminary experiment using WYHV antiserum.

^b GHV = number of times greater (calculated by dividing the ELISA value by that of the healthy control, which is shown as 1.0) than the healthy control.

^c The RHBV-positive control was from lyophilized rice leaf tissue. Tissue was weighed and assumed 90% water loss due to lyophilization; then, the calculated weight was used to approximate a 1:30 wt/vol dilution used for the Kansas and North Dakota samples.

Table 4. Average enzyme-linked immunosorbent assay (ELISA) values against antiserum to Wheat yellow head virus (WYHV) and *Rice hoja blanca virus* (RHBV) from either symptomatic (S) or nonsymptomatic (NS) heads (H) and flag leaves (F) of naturally infected wheat collected in 2001 from South Dakota and in 2002 from North Dakota and Oklahoma (heads sampled only) or healthy wheat (HW)^a

Tissue	South Dakota		North Dakota				Oklahoma			
	WYHV		WYHV		RHBV ^b		WYHV		RHBV ^b	
	S	NS	S	NS	S	NS	S	NS	S	NS
H	0.120 (11)	NV (0)	0.163 (15)	NV (0)	0.205 (15)	NV (0)	0.192	NV (0)	0.368 (15)	NV (0)
F	0.201 (6)	0.024 (5)	0.256 (12)	0.026 (3)	0.142 (9)	0.017 (3) ^c	NT	NT	NT	NT
HW-H	...	0.010	...	0.007	...	0.010	...	0.031	...	0.026
HW-F	...	0.009	...	0.008	...	0.015	...	NT	...	NT
WYHV	0.139		0.337		0.094		0.181		0.331	

^a In all, 11 plants were tested for the South Dakota sample and 15 each for the North Dakota and Oklahoma analyses. Number in parenthesis following each ELISA value indicates the number of plants whose ELISA values were used to calculate the average ELISA value; NV = no value because no tissue was present with the indicated symptom type; NT = not tested.

^b No positive control was available for RHBV for the Oklahoma and South Dakota ELISA analyses. However, previous work where RHBV controls were available demonstrated the specificity of the RHBV antiserum for RHBV. The North Dakota samples were not positive for *Wheat streak mosaic virus* (WSMV) or the High Plains virus but eight of the Oklahoma samples were infected with WSMV as well. Oklahoma samples positive in ELISA for WSMV had ELISA values ranging from 3.5 to 16.6 times larger than the healthy wheat control.

^c For the North Dakota RHBV sampling, three flag leaves with symptoms recorded low values of 0.038, 0.045, and 0.021, while the same leaves tested strongly against the WYHV antiserum with values of 0.434, 0.325, and 0.304, respectively. The latter three ELISA values were not used in determining the 0.017 value in the table for the NS tissue sample, nor were they used to calculate the 0.142 value.

kota, and Oklahoma sources) having YHD symptoms, but not to WSMV and HPV (Table 1–4). These results further weakened the argument that the pathogen inciting yellow head symptoms in wheat might be a variant of HPV, which also has an N-protein of approximately 32 kDa (16). However, the possibility still could not be excluded that the WYH disease agent might be an HPV variant not reactive to HPV antiserum but capable of inciting yellow head symptoms.

The analysis by TOFMS of the unique protein associated with the pathogen transferred from wheat with YHD symptoms showed that the agent was not a, HPV variant, but possessed a protein most closely related to the N-protein of RHBV (7; Fig. 2). Therefore, the protein observed in wheat with YHD symptoms and subsequently transmitted to maize and *N. benthamiana* was an N-protein of viral origin. However, the WYHV amino acid sequence differed from that of RHBV by 32% (Fig. 2). This value falls outside the 15% limit set to delimit species parameters for corresponding nucleocapsid gene products required for establishing a new virus species (23). The evidence we have adduced supports our suggestion to name the agent that incites YHD symptoms in wheat as Wheat yellow head virus.

The calculated molecular weight determined by TOFMS of the WYHV N-protein was 35,373 (including N-terminal acetylation), which was close to the range (32 to 34 kDa) we observed using SDS-PAGE. The greater accuracy and precision of TOFMS for determining mass of virus proteins has been demonstrated previously for *Brome mosaic virus* (36), *Foxtail mosaic virus* (30), *Zea mosaic virus* (35), and *Johnsongrass mosaic virus* (28). The N-proteins of tenuiviruses have a relative molecular weight of 34 to 35 kDa (23), which is in the range we report by SDS-PAGE and TOFMS for the N-protein of WYHV.

The relationship of the RHBV and WYHV N-protein amino acid sequence suggests that WYHV might be derived from RHBV. RHBV first was reported in the United States in 1957 in Florida (1), in 1958 in Mississippi (2), and in 1960 in Louisiana (3), allowing about 50 years of evolutionary divergence and selection for the specific niche now occupied by WYHV. Phylogenetic analysis of RNA-3 (N-protein) and RNA-4 (MNP protein) of RHBV, EHBV, and UHBV has been used to deduce that the evolution of EHBV and UHBV from an ancestral form of RHBV was caused by introduction of new hosts (8). It is interesting to note that comparison of the coding region of RNA-3 (N-protein) of RHBV, EHBV, and UHBV showed that EHBV and UHBV have a 81.2 and 81.6% similarity to RHBV, respectively (8), whereas WYHV has 68% similarity to RHBV. Further support for a hypothesis of

evolutionary divergence and selection in new niches might be found in the interesting report that Colombian and Costa Rican strains of RHBV are over 90% identical (9). It also is possible that the WYHV was present in 1957, because the work reporting RHBV was conducted for rice and was based on symptom expression (1), although vector identity (*Tagosodes orizicolus*) and symptoms were reported in the Mississippi (2) and Louisiana (3) identifications. RHBV has been reported to infect wheat, but no yellow head symptoms were described for the infected wheat, and leaf symptoms were described ranging from mottling to stripping and severe chlorosis (14,15,19). In contrast, the leaf symptom reported here was a mosaic on wheat infected by WYHV in Kansas. A mosaic symptom also was present on samples that reacted to WYHV antiserum from Oklahoma, North Dakota, and South Dakota.

An asymmetric response was observed between WYHV and RHBV antiserum in both ELISA and Western blotting tests (Fig. 3B and C; Table 3). In these tests, WYHV antiserum did not react to RHBV but RHBV antiserum reacted to WYHV. We have noted such asymmetric serological responses to *Zea mosaic virus* (ZeMV) and *Sugarcane mosaic virus* strain MDB (SCMV-MDB), where ZeMV antiserum showed a stronger response to SCMV-MDB than did SCMV-MDB antiserum to ZeMV (35).

The analyses of Western blots probed with anti-RHBV-MNP (Fig. 3D) demonstrate the presence of WYHV proteins migrating to the same position (approximately 16 kDa) as those for RHBV (10). The lack of, or faint bands of, the 16 kDa proteins in maize extracts from WYHV-infected tissue (Fig. 3D) and *N. benthamiana* (Fig. 3D) may reflect either lower levels of this protein produced in these species than wheat, or problems in purifying this protein from these species, or both. The single band immediately below the 16-kDa bands in lanes 2 and 3 probably is a breakdown product associated with the MNP. In addition, our results demonstrated a robust response in ELISA to the RHBV MNP antiserum with WYHV and NDV extracts (Table 3). The antiserum to RHBV MNP we used in our tests has been demonstrated previously to react only to the 16-kDa protein of RHBV and EHBV (10). The 16-kDa MNP is present in several plant viruses, including EHBV (10), *Maize stripe virus* (10), RHBV (10), *Rice stripe virus* (11), and UHBV (8) and is classified in the genus *Tenuivirus* (23).

ELISA analysis of wheat from Oklahoma, North Dakota, and South Dakota showed that plants with yellow head symptoms and symptoms on the flag leaves reacted to WYHV virus antiserum and to RHBV antiserum. This indicates that the distribution of WYHV or a closely related virus reacting to these antisera exists in the

Great Plains. In samples from Kansas as well as those from Oklahoma, North Dakota, and South Dakota, symptomatic tissue was largely confined to yellow heads and mosaic on the flag leaf. This could indicate a late infection, resulting in symptoms in only these tissues. The pattern of symptom expression in rice infected by RHBV in Louisiana was also stated to be confined to young top leaves and the panicle in rice (3).

We were not able to transmit the 1997 isolate of WYHV using aphids or leafhoppers. The relationship of WYHV to RHBV suggests that the best candidate for WYHV vector would be a planthopper, because RHBV is vectored by a planthopper (*Sogata orizicola* (Muir) = *Tagosodes orizicolus*; 13). This may explain the lack of transmission to wheat by the aphids and leafhoppers tested early in our studies. Unfortunately, the loss of WYHV transmissibility following determination of the sequence prevented subsequent acquisition experiments with planthoppers. However, sweeps of grass and wheat were made in May and June 2001 and two species of planthoppers were collected that included *Delphacodes campestris* (Van Duzee) and *D. lutulenta* (Van Duzee). However, wheat infested with these planthoppers did not become symptomatic following infestation and tested negative in ELISA against WYHV and RHBV antiserum. No information is available on the presence of the vector of RHBV (*Tagosodes orizicolus*) in Kansas. Because 24 h of exposure at 6 to 7°C or below kills eggs and all forms of this planthopper (43), survival in the state is unlikely. Thus, it is probable that other planthopper species may serve as the vector and may be responsible for the infection of wheat in Kansas, Oklahoma, and North and South Dakota. The large 32% difference in sequence of the N-protein of WYHV from that of RHBV also might suggest that different leafhopper vectors for WYHV are involved although, at present, there are no data showing that the N-protein accounts for vector specificity for RHBV, EHBV, and UHBV or any known tenuiviruses. Also, it has been suggested that extreme feeding preference of a respective planthopper is important in the acquisition of RHBV and EHBV (8,12,19). Although the major vector for RHBV was reported as *T. orizicolus* (23) and for EHBV as *T. cubanus* (23), it has been shown that *T. cubanus* can, in fact, acquire and vector RHBV by forced feeding on RHBV-infected rice (12). More than one species could be involved as a WYHV vector; therefore, host preference may be of major importance. However, in other virus-vector relationships, such as *Cucumber mosaic virus*, single amino acid changes in the coat protein have been shown to differentially affect transmission by aphids (24).

The tenuiviruses are not mechanically transmissible and the host range is re-

stricted to members of *Graminae* (23). However, we observed that WYHV was infective by mechanical inoculation to *N. benthamiana*, as was the KSU00-105 isolate and the NDV. The infection of a plant species not belonging to the family *Gramineae* may be unique to WYHV. *N. benthamiana* may be susceptible to infection by RHBV and other viruses of the genus *Tenuivirus*, but it has not been tested.

In conclusion, the sum of our results demonstrates that a virus is the causal agent of YHD of wheat in Kansas and that it, or a serologically related virus, also is present in wheat with YHD symptoms in Oklahoma, North Dakota, and South Dakota. Although wheat with YHD occurs in several states in the Great Plains, the economic effects of YHD do not appear to be significant. However, it would be well to accurately identify the vector or vectors of WYHV, the natural host range, and the genomic organization and expression of the virus. With this information, it would be easier to monitor the evolution of this disease and fashion appropriate control strategies should YHD become of greater economic importance, a development that might come about as new wheat cultivars are deployed and changing environments favor the vector or vectors of WYHV.

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