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Genetic Transformation System for the Fungal Soybean Pathogen *Cercospora kikuchii*

R. G. UPCHURCH,[1]* M. EHRENSHAFT,[1] D. C. WALKER,[2]† AND L. A. SANDERS[2]‡

Agricultural Research Service, U.S. Department of Agriculture,[1] and Department of Plant Pathology,[2] North Carolina State University, Raleigh, North Carolina 27695-7616

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An altered β-tubulin gene that confers resistance to the fungicide benomyl was isolated from a genomic library of a UV-induced mutant of *Cercospora kikuchii* and used as a selectable marker for transformation. The level of benomyl resistance conferred to the transformants was at least 150-fold greater than the intrinsic resistance of the *C. kikuchii* recipient protoplasts. In the majority of cases, the tubulin fragment was integrated at the native β-tubulin locus, apparently by gene replacement or gene conversion. The frequency of transformation ranged from 0.2 to 6 transformants per μg of DNA, depending on the recipient strain. Transformation with linearized plasmid resulted in a higher frequency, without changing the type of integration event. Transformants were phenotypically stable after eight consecutive transfers on medium without benomyl. This is the first report of a genetic transformation system for a *Cercospora* species.

The filamentous fungus *Cercospora kikuchii* is a pathogen of soybean and the causative agent of the economically important disease known as purple seed stain (16). Purple seed stain is the most widely distributed fungal disease of soybean seed, and no immune or highly resistant commercial soybean cultivars are known (16). Many species of *Cercospora* (8), including *C. kikuchii* (10), produce the non-host-specific, red polyketide toxin cercosporin. Cercosporin is a photosensitizing compound (2) known to cause membrane damage and cell death by the peroxidation of membrane fatty acids (2). It is postulated that tissue colonization and nutrient acquisition by the pathogen are facilitated by the action of this necrosis-inducing phytotoxin. Inoculation of soybean with cercosporin-blocked mutants of *C. kikuchii* has shown that the production of cercosporin is a crucial pathogenicity factor (19).

More-effective disease control methods can be developed once the biochemistry and genetics of cercosporin biosynthesis are better understood. Although the structure (11, 23) and chemical mechanism of action of cercosporin are known (2), no pathway intermediates, enzymes, or genes have been isolated (13). One approach to identifying cercosporin genes requires the development of a genetic transformation system. We have recently obtained mutants altered in the accumulation of cercosporin (19). The availability of blocked mutants together with a genetic transformation system would allow the identification of genes by functional complementation, either by direct screening of a wild-type genomic library or by the transformation of selected, single clones into mutant recipients (22).

Positive selection of transformants by complementation of auxotrophic mutants to prototrophy requires that recipient strains have the appropriate auxotrophic mutation (6). For *C. kikuchii* and other *Cercospora* species that lack both an identified teleomorph and a parasexual system for genetic manipulation, this requirement poses difficulties when several recipient strains are used. The use of dominant selectable markers, such as metabolic inhibitors, obviates the need for prior recipient modification. Unfortunately, it was not possible to use a metabolic inhibitor such as hygromycin (6) because of the insensitivity of *C. kikuchii* protoplasts to this agent. Resistance to the fungicide benomyl has been used as a dominant selectable marker in other fungi (6); however, the benomyl resistance genes from *Aspergillus nidulans*, *Aspergillus flavus*, and *Neurospora crassa* are apparently not expressed in *C. kikuchii* at levels sufficient for selection of transformants (18).

The use of homologous marker genes has increased transformation efficiencies in some systems (6). This study was therefore initiated to isolate a native β-tubulin gene from a benomyl-resistant strain of *C. kikuchii* and to use it as a selectable marker for transformation. In this report, we describe the development of a transformation system for *C. kikuchii* in which we used a homologous β-tubulin gene that was isolated from a genomic library of a benomyl-resistant mutant.

MATERIALS AND METHODS

Fungal strains and cultures. *C. kikuchii* wild-type strain PR, which can be induced to sporulate and produces cercosporin on laboratory media, was obtained from J. B. Sinclair (University of Illinois, Urbana). *C. kikuchii* PRBn^r is resistant to the fungicide benomyl, whose active ingredient is 2-(methoxycarbonylamino)benzimidazole (MBC; a gift of DuPont, Wilmington, Del.) at ≥10 μg/ml. Mutant PRBn^r was obtained from UV-irradiated conidia of PR as previously described (19). Cultures of *C. kikuchii* were maintained on minimal medium and complete medium (CM) on agar and in liquid form (19). Benomyl was added to media from a 1-mg/ml stock solution in dimethyl sulfoxide to give final medium concentrations ranging from 10 ng/ml to 10 μg/ml. Cultures were incubated at 25°C.

DNA preparation. *Escherichia coli* DH1 and JM 109 were used in this study. Bacterial culture, transformations, and DNA preparations were done by standard methods (12). pGEM cloning vectors were purchased from Promega. High-molecular-weight fungal DNA (estimated size, >150 kb) was

* Corresponding author.
† Present address: Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624.
‡ Present address: Department of Biology, Tulane University, New Orleans, LA 70118.

isolated from *C. kikuchii* by the method of Garber and Yoder (7).

Construction and screening of the genomic libraries. Cosmid pAF1 (22) was kindly provided by C. P. Woloshuk, N.C. State University, Raleigh. The genomic library was constructed as described for other filamentous fungi (15). Insert DNA was prepared by partial digestion of *C. kikuchii* DNA with *Sau*3A. The DNA (400 μg) was size fractionated on a 1 to 5 M NaCl gradient by ultracentrifugation at 25,000 rpm for 12 h in a Beckman SW-28 rotor at 20°C. DNA ranging in size from 35 to 45 kb was used for ligation into pAF1. The cosmid vector pAF1 was digested to completion with *Bam*HI and then dephosphorylated with calf intestinal alkaline phosphatase. Insert DNA (2 μg) was ligated with 3 μg of dephosphorylated vector DNA in a 10-μl volume. A 3-μl sample of the ligation mixture was treated with a lambda packaging extract supplied by Boehringer Mannheim Biologicals. The packaged cosmids were used to transfect *E. coli* DH1 as described above, resulting in 2.6×10^4 ampicillin-resistant colonies per μg of DNA. A random plasmid screening of 16 of these colonies revealed an average insert size of approximately 38 kb.

A total of 1,423 colonies were screened by standard colony blot hybridization (11). A 2.58-kb *Hin*dIII-*Sal*I fragment from pSV50 (14, 21) (a gift of C. Yanofsky, Stanford University) that encodes a benomyl-resistant form of β-tubulin from *N. crassa* was used as a probe to screen the library.

Fungal transformation. Mycelia (0.1 ml of a suspension of a single 5-mm-diameter plug disrupted in 3 ml of water) were inoculated into 200 ml of CM medium and grown in shake culture (180 rpm) for 5 days at 25°C. On day 5, the culture was blended (two 8-s cycles) in a Waring blender, and 50 ml of the mycelial slurry was inoculated into 200 ml of fresh CM and incubated overnight. To prevent potential damage by cercosporin to protoplasts, wild-type (cercosporin-producing) PR cultures were subcultured before significant cercosporin accumulation had occurred (day 3) or grown in continuous darkness (achieved by wrapping culture flasks in foil and dark cloth) for 5 days, a condition under which no significant cercosporin is produced (19). One gram (wet weight) of mycelium was completely resuspended in 40 ml of an enzyme solution containing Novozyme 234 (5 mg/ml; Novo Nordisk Biolabs), β-glucuronidase (1,200 U/ml; Sigma), 0.7 M NaCl, 10 mM $CaCl_2$, and 10 mM $NaPO_4$ (pH 5.8). After digestion at 30°C for 3 h, protoplasts were filtered, in succession, through cheesecloth, glass wool, and a 30-μm-mesh screen. Protoplast suspensions ($\geq 10^8$ protoplasts per ml), free of mycelial debris, were routinely achieved by this method. Subsequent washing, resuspension, and transformation procedures were exactly as described for the transformation of *Cochliobolus heterostrophus* (17). Plasmid DNA (5 μg), either in circular form or linearized by endonuclease restriction, was used to transform 10^7 protoplasts in 100 μl. Protoplasts were regenerated at 25°C for 7 to 10 days in either minimal medium or CM agar containing 1 M sucrose, with and without 100 ng of benomyl per ml. Of the 10^7 viable protoplasts in the transformation mixture, approximately 20% were recovered on regeneration medium, as determined by colony counts.

Southern hybridization analysis of transformants. Fungal and plasmid DNAs were digested to completion by restriction endonucleases, electrophoresed through 0.8% agarose, and blotted to nitrocellulose. Filters were first hybridized with a ^{32}P-labeled 3.5-kb *Sal*I β-tubulin fragment of *C. kikuchii* and, after this probe was stripped off, hybridized with radiolabeled pGEM3zf+ vector (5, 12).

Stability and radial growth. To determine the mitotic stability of the transformants, hyphal tips from transformant colonies were transferred eight times in succession on CM medium without benomyl. After each transfer to nonselective medium, a 5-mm-diameter plug from the colony periphery was plated on CM medium with various concentrations of benomyl. The radial growth of each consecutive transfer was determined.

RESULTS

Isolation and subcloning of the *C. kikuchii* β-tubulin gene. The genomic library of *C. kikuchii* PRBn^r was screened with the β-tubulin gene from *N. crassa*. Of 1,423 colonies tested, one clone, designated pCKB1, hybridized strongly to the tubulin probe. Analysis of *Eco*RI and *Bgl*III restriction digests of pCKB1 and genomic DNA showed that a single fragment hybridized to the tubulin probe, indicating that the region of homology lacked internal restriction sites for these enzymes (data not shown).

An *Eco*RI restriction fragment of approximately 17 kb containing the altered β-tubulin gene was subcloned from pCKB1 into pGEM11zf+. Southern hybridization analysis and restriction mapping of this *Eco*RI fragment revealed that a single, internal 5.2-kb *Xho*I fragment (Fig. 1A) hybridized to the *N. crassa* tubulin probe (data not shown). This 5.2-kb *Xho*I fragment and a 400-bp *Pst*I *cos* fragment (from cosmid pAF1) were then ligated into the *Sal*I and *Pst*I cloning sites of pGEM3zf+, respectively. The resulting *C. kikuchii* cosmid transformation vector was designated pCKB4 (Fig. 1). Subsequent mapping and hybridization studies have shown that the sequence with homology to the *N. crassa* tubulin probe is an approximately 2.1-kb *Xho*I-*Bgl*II fragment of pCKB4.

Transformation and expression of the *C. kikuchii* β-tubulin gene. *C. kikuchii* PR and cercosporin mutants U1 to S2 (19) were transformed with pCKB1 and pCKB4. Transformants were selected directly on medium containing 100 ng of benomyl per ml. Untransformed protoplasts of wild-type *C. kikuchii* PR and the cercosporin mutants were killed by benomyl at concentrations ranging from 20 to 60 ng/ml (data not shown), while the benomyl-resistant mutant, PRBn^r, was insensitive to concentrations of the fungicide greater than 10 μg/ml. Clear differentiation of untransformed and benomyl-resistant colonies was achieved by the incorporation of 100 ng of benomyl per ml of molten agar medium (Fig. 2). All U2 transformants in this study were resistant to benomyl at concentrations ≥10 μg/ml. Unlike authentic transformants, the slowly growing colonies infrequently detected on control plates were not viable when transferred to fresh medium containing benomyl. Spontaneous revertants to benomyl resistance were not detected on control plates in any of the experiments. Transformation efficiencies varied with recipient strain and with plasmid vector and according to whether the plasmid was supplied in circular or linearized form (Table 1). Transformation frequency with the genomic cosmid pCKB1 ranged from 0.8 to 6 transformants per μg of DNA for mutants S2 and U2, respectively. Transformation frequencies with circular pCKB4 were uniformly low, 0.2 to 0.8 transformant per μg, for all recipients. When pCKB4 was linearized by restriction of the *Sma*I site (outside of the tubulin sequence), frequencies ranged from 0.6 to 6 transformants per μg for S1 and PR, respectively, depending on the recipient.

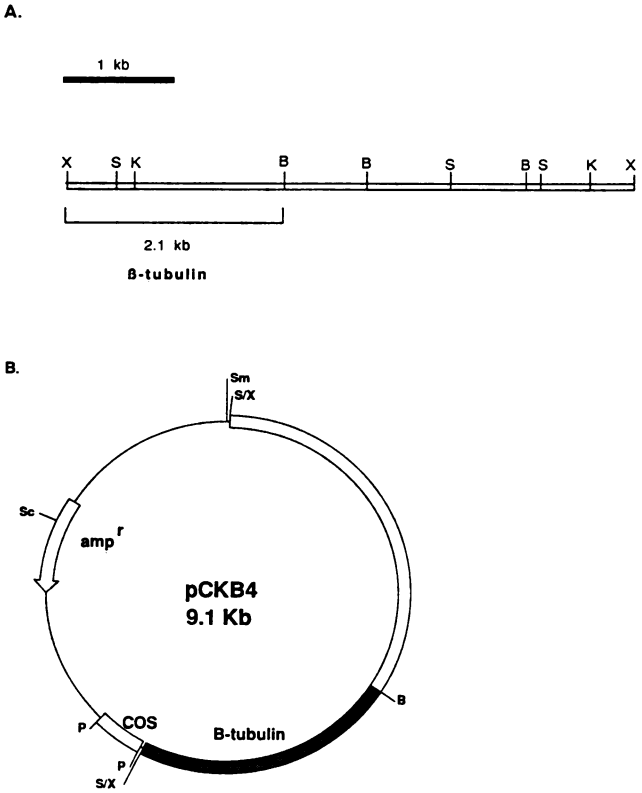

FIG. 1. (A) Restriction endonuclease site map of the 5.2-kb *Xho*I DNA fragment of *C. kikuchii* PRBn^r that hybridized to the 2.58-kb *Hin*dIII-*Sal*I *N. crassa* β-tubulin gene probe (see text). Restriction endonuclease sites: B, *Bgl*II; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sc, *Sca*I; Sm, *Sma*I; X, *Xho*I. (B) Restriction endonuclease map of cosmid vector pCKB4 (9.1 kb) used for transformation of benomyl-sensitive *C. kikuchii* recipients. A 400-bp *Pst*I lambda *cos* fragment and the 5.2-kb *Xho*I fragment containing the altered tubulin gene were ligated into the *Pst*I and *Sal*I sites of pGEM3zf+ (Promega), respectively. The shaded region indicates the 2.1-kb *Xho*I-*Bgl*II fragment from *C. kikuchii* that hybridized to the β-tubulin gene from *N. crassa*.

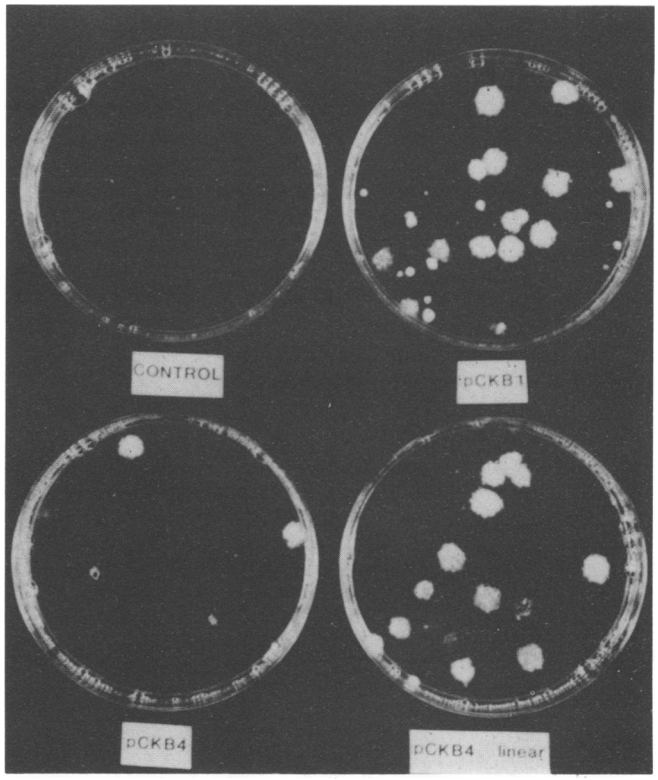

FIG. 2. Growth of benomyl-resistant *C. kikuchii* U2 transformants on regeneration medium containing 100 ng of benomyl per ml. Protoplasts were transformed with either the genomic library cosmid pCKB1 or cosmid vector pCKB4 in circular or linearized form (see text). Control transformations were done with no plasmid DNA.

Hybridization analysis of transformants. Southern analysis of untransformed PR, PRBn^r, the U2 recipient, and U2 transformants with tubulin and vector probes is shown in Fig. 3. Analysis of PR, PRBn^r, and the U2 recipient (lanes 1, 2, and 3, respectively) with the tubulin probe showed hybridization to the single, native 5.2-kb *Xho*I fragment.Stripping of this blot (Fig. 3B) and reprobing with vector sequences did not detect vector-hybridizing fragments.

Two patterns of hybridization were found for the transformants. In 11 of the 14 U2 transformants, the tubulin probe hybridized to a single 5.2-kb *Xho*I fragment indistinguishable from the tubulin gene-containing fragment of the recipient (Fig. 3A). None of these transformants had sequences with homology to the vector (Fig. 3B). The other three U2 transformants (lanes 5, 11, and 15) had an additional 7.6-kb fragment that hybridized to both the tubulin and vector probes. Seven U2 transformants transformed by circular pCKB1 were also analyzed by Southern blots in the same manner as the transformants for which results are shown in Fig. 3 (data not shown). A single 5.2-kb *Xho*I fragment hybridized to the tubulin probe with no vector hybridization in five of the seven transformants, and two of the transformants had an additional 7.6-kb fragment hybridizing to both tubulin and vector probes. This hybridization pattern is exactly like that of pCKB4 transformants in lanes 5, 11, and 15 of Fig. 3.

Mitotic stability of the transformants. A total of 21 U2 transformants, including the 14 analyzed by Southern hybridization (Fig. 3), were assessed for mitotic stability of benomyl resistance. After each consecutive transfer on nonselecting medium, including the eighth and final one, all transformants were resistant to benomyl at a concentration of ≥10 μg/ml, with no inhibition of growth. In contrast, the

TABLE 1. Transformation frequencies for *C. kikuchii* wild-type PR and cercosporin mutants transformed with circular and linear plasmids containing the *C. kikuchii*-altered β-tubulin gene that confers resistance to benomyl

Strain	Avg no. of transformants/μg of DNA (no. of expts)		
	Circular pCKB1	Circular pCKB4	Linear pCKB4
PR	3 (3)	0.8 (2)	5 (2)
U1	ND[a]	0.6 (2)	2 (2)
U2	6 (10)	0.8 (5)	4 (4)
U4	1 (2)	0.2 (2)	0.8 (2)
S1	ND	0.2 (2)	0.6 (2)
S2	0.8 (3)	0.6 (4)	0.8 (4)

[a] ND, not determined.

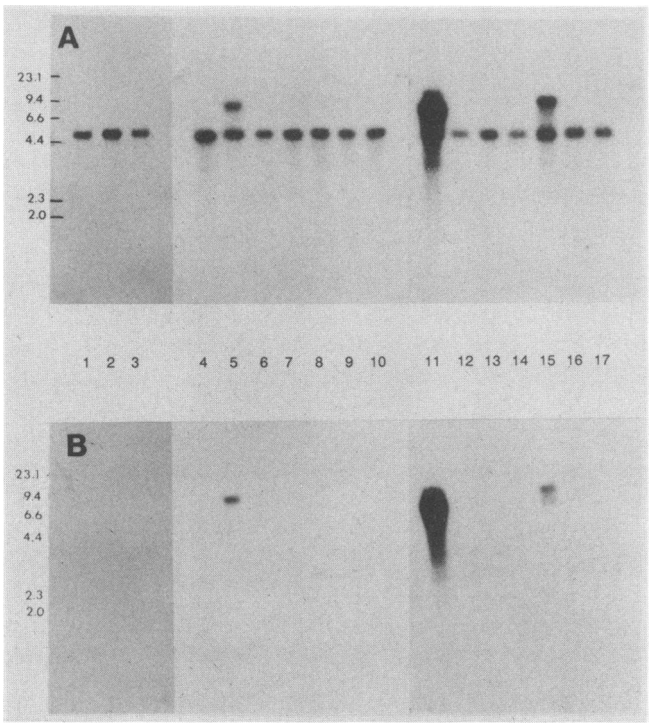

FIG. 3. Southern hybridization analysis of 14 *C. kikuchii* U2 transformants. Genomic DNA (5 μg per lane) from untransformed *C. kikuchii* wild-type PR, PRBn^r, and cercosporin mutant U2 (lanes 1 through 3, respectively) and U2 transformed with either circular pCKB4 (lanes 4 through 10) or linearized pCKB4 (lanes 11 through 17) was digested with *Xho*I and electrophoresed through a 0.8% agarose gel. DNA was blotted onto nitrocellulose and probed with a ^{32}P-labeled 3.5-kb *Sal*I *C. kikuchii* tubulin fragment (A) and ^{32}P-labeled vector pGEM3zf+ (B). Molecular sizes in kilobases are indicated on the left.

U2 recipient was sensitive to as little as 40 ng of benomyl per ml.

DISCUSSION

We have isolated a genomic DNA fragment containing a *C. kikuchii*-altered β-tubulin gene, incorporated the gene into a cosmid vector, and demonstrated that protoplasts of both wild-type and cercosporin-altered mutants are transformed to benomyl resistance by this gene. A transformation system based on the homologous tubulin gene was developed because the expression of heterologous tubulin gene-based vectors was undetectable in *C. kikuchii*. In this report, we have described the first transformation protocol for a *Cercospora* species.

Transformation of filamentous fungi typically results in a phenotypically complex variety of transformants including the abundant class of transformants with the selected marker integrated at ectopic and multiple sites (1, 6). However, variation in the level of benomyl resistance in *C. kikuchii* transformants was not observed. *C. kikuchii* protoplasts, exquisitely sensitive to benomyl (MIC, 20 to 60 ng/ml), were transformed to uniformly high levels of resistance (≥10 μg/ml). This is in contrast to the multiple levels of resistance observed for *A. flavus* transformed by a vector containing both the *pyr-4* and the homologous β-tubulin genes (15). The most benomyl-resistant *A. flavus* transformant class had multiple copies of the β-tubulin gene incorporated into multiple sites in the genome (15). In contrast, unexpectedly, in 11 of 14 *C. kikuchii* pCKB4 transformants (Fig. 3A) and 5 of 7 pCKB1 transformants (data not shown), integration of the altered tubulin gene occurred only at the resident locus. Integration of the altered tubulin fragment at the native tubulin site would usually be expected to result in a larger hybridizing fragment (3). However, alteration of fragment size in these 16 U2 transformants was not observed. Since hybridization to the tubulin fragment but not to vector (Fig. 3B) was found, and since the size of the hybridizing tubulin fragment was similar to that of the recipient DNA, it appears that the predominant recombinational event was gene replacement of the resident wild-type tubulin sequence or possibly gene conversion. This pattern of recombination in *C. kikuchii*, though less common in filamentous fungi (6), is similar to the high frequencies of gene replacement observed for *Aspergillus niger* transformed with a vector containing the homologous *pyrG* gene (20). High frequencies of recipient-dependent gene conversion have also been reported for transformation of *N. crassa* with the *trp-1* gene (9).

Southern analysis for three pCKB4 transformants (Fig. 3, lanes 5, 11, and 15) and also two of seven pCKB1 transformants (data not shown) revealed an additional, ectopic 7.6-kb *Xho*I fragment that hybridized to both the tubulin and the vector probes. This suggests that heterologous integration of a fragment containing truncated vector and/or tubulin sequences occurred in these five transformants. No transformants exhibiting homologous integration (single crossover events) were detected in the analysis of these 21 independent transformants.

We transformed *C. kikuchii* U2 with circular tubulin vectors containing inserts of 37 kb (pCKB1) and 5.2 kb (pCKB4) and observed frequencies of 5 to 6 and 0.8 transformants per μg of DNA, respectively. Our results are in agreement with general observations. For example, transformation of *N. crassa* auxotrophic mutants by *am* (glutamate dehydrogenase) gene vectors showed that the frequency of homologous integration at the *am* locus appears to be related, in part, to the length of chromosomal homology provided in the insert (1). Linearization has been reported to increase the transformation frequency of the selectable marker in several systems (6), but the explanation for this remains unclear. We observed a fivefold increase in transformation frequency when U2 was transformed with linear pCKB4 (Table 1).

Although the low transformation frequency and preponderance of apparent gene replacement and/or gene conversion events characteristic of this system could indicate that an incomplete tubulin gene was cloned, hybridization evidence and the strong homology among tubulin genes give us a high degree of confidence that the mutated tubulin gene of *C. kikuchii* is represented in its entirety in the 37-kb insert of genomic cosmid pCKB1, in the 17-kb *Eco*RI fragment subcloned from pCKB1, and in the internal 5.2-kb *Xho*I fragment subcloned from the 17-kb *Eco*RI fragment. Only the *Eco*RI and *Xho*I fragments hybridized when restriction fragments and genomic DNA digested with *Eco*RI or *Xho*I were probed with the complete *N. crassa* tubulin gene. Although we have shown only the 5.2-kb *Xho*I fragment map in Fig. 1, this fragment is an internal fragment of the 17-kb *Eco*RI cosmid subclone and thus an internal subclone of the pCKB1 insert. Low frequencies of transformation are not unusual among the transformation systems reported for plant pathogens, as illustrated by the low frequency of

transformation of the homologous tubulin vector developed for *A. flavus* (15).

The ability to use the altered β-tubulin gene permits the transformation of *C. kikuchii* isolates without prior mutation of recipients to auxotrophy. Although the low frequency of transformation precludes the use of this selective marker for screening a genomic library, the frequency is high enough to use in attempts to complement mutants with specific clones. An unusual feature of our system is the high frequency of homologous recombination (apparently gene replacement or gene conversion) in *C. kikuchii* recipients. This feature may allow us to target incoming genes to their native genomic loci and to carry out precise gene disruption studies. A cosmid genomic library of *C. kikuchii* wild-type PR has been constructed by ligation of DNA partially digested with *Hae*III into the *Sma*I site of pCKB4. Genomic cosmids from this library corresponding to two cDNAs whose message accumulation is light enhanced and temporally parallels cercosporin production (4) have been isolated.

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