Duplication of a Conditionally Dispensable Chromosome Carrying Pea Pathogenicity (*PEP*) Gene Clusters in *Nectria haematococca*

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A supernumerary chromosome called a conditionally dispensable chromosome (CDC) is essential for pathogenicity of *Nectria haematococca* on pea. Among several CDCs discovered in *N. haematococca*, the *PDA1* CDC that harbors the pisatin demethylation gene *PDA1* is one of the best-studied CDCs and serves as a model for plant-pathogenic fungi. Although the presence of multiple copies is usual for supernumerary chromosomes in other eukaryotes, this possibility has not been examined well for any CDCs in *N. haematococca*. In this study, we produced strains with multiple copies of the *PDA1* CDC by protoplast fusion and analyzed dosage effects of this chromosome. Using multiple methods, including cytological chromosome counting and fluorescence in situ hybridization, the fusion products between two transformants derived from the same strain that bears a single *PDA1* CDC were shown to contain two *PDA1* CDCs from both transformants and estimated to be haploid resulting from the deletion of an extra set or sets of A chromosomes in the fused nuclei. In phenotype assays, dosage effects of *PDA1* CDC in the fusion products were evident as increased virulence and homoserine-utilizing ability compared with the parents. In a separate fusion experiment, *PDA1* CDC accumulated up to four copies in a haploid genome.

Additional keywords: FISH, *Fusarium solani*, germ tube burst method.

Since its introduction in the early 1980s, the wide application of pulsed-field gel electrophoresis (PFGE) has revealed that intraspecific chromosome polymorphisms are very common in fungi (Mills and McCluskey 1990; Zolan 1995). Such polymorphisms take the form of variation in chromosome size or number between strains, in which not only ordinary (or A chromosomes) but also supernumerary chromosomes similar to “B” chromosomes known in plants and animals are involved. Interestingly, some supernumerary chromosomes in plant-pathogenic fungi differ from common supernumerary or B chromosomes in that they contain functional genes that play important roles in causing disease on a specific host (Covert 1998; Hatta et al. 2002; Johnson et al. 2001). Thus, those chromosomes that provide adaptive advantages in certain habitats, such as the ability to cause disease, were referred to as conditionally dispensable chromosomes (CDCs) (Covert 1998).

In mating population (MP) VI of *Nectria haematococca* Berk. & Broome (anamorph: *Fusarium solani*) pathogenic on pea, at least five supernumerary chromosomes have been identified (Covert 1998). Of these, two 1.6-megabase (Mb)-sized chromosomes, named *MAK1 PDA6-1* CDC and *PDA1* (also called *PDA1-1*) CDC to reflect the phytoalexin-detoxifying genes on these chromosomes, qualified as CDCs based on their proven dispensability (Miao et al. 1991; VanEtten et al. 1998) and the occurrence of functional genes involved in pathogenicity (Covert et al. 1996; Miao et al. 1991). So far, the structure and function of these two CDCs have been well characterized. As far as molecular aspects, for instance, phytoalexin-detoxifying genes have been cloned (Covert et al. 1996; Maloney and VanEtten 1994), transposon-like elements detected (Kim et al. 1995; Shiflett et al. 2002), a physical map constructed (Enkerli et al. 2000), and the pea pathogenicity (*PEP*) gene cluster (*PEP* cluster) characterized (Han et al. 2001). In addition, as part of the whole genome sequencing project using a strain with *PDA1* CDC that is now close to completion, an estimated 17 chromosomes for this pathogenic fungus have been found (Joint Genome Institute, *N. haematococca*, v.2).

In addition to these molecular biological studies, the inheritance and cytological characteristics of these chromosomes also have been analyzed. The data obtained for the *MAK1 PDA6-1* CDC indicated that it frequently behaves in a non-Mendelian manner during sexual propagation (Miao et al. 1991) and that its morphological appearance in mitotic metaphase is similar to that of A chromosomes (Taga et al. 1999).

Despite such progress, many important issues still need to be addressed for the CDCs of *N. haematococca*, including *MAK1 PDA6-1* and *PDA1* CDCs. Among others, the accumulation of CDCs in multiple copies in the genome might produce more virulent strains, and the transfer of CDCs to strains of other species that lack the CDCs might transform nonpathogenic strains of pea into pathogenic strains. Concern about these issues is reasonable because accumulation in the genome is a property common to supernumerary chromosomes in various organisms (Camacho 2005; Jones 1991) and interstrain transfer of the fungal supernumerary chromosome in *Colletotrichum gloeosporioides* was shown to occur in a laboratory (He et al. 1998) and in nature for *Alternaria alternata* (Masunaka et al. 2005). Unfortunately, it is difficult with the currently available techniques to assess whether accumulation and transfers actually happen in *N. haematococca* in fields. Therefore, it should be rewarding to examine the potential of such cases to occur under laboratory conditions.

In this study, we analyzed *PDA1* CDC, focusing on its accumulation in the genome of a standard strain. Our strategy was to exploit protoplast fusion for forcing chromosome accumula-

tion in combination with cytological chromosome visualization for analyzing genome components. Consequently, we succeeded in accumulating more than two *PDA1* CDCs in a haploid genome and could examine the effects of the disomic CDC on the phenotypes of host fungus. Although the artificial deletion and truncation of the *PDA1* CDC were reported previously in *N. haematococca* (Kistler and Benny 1992; VanEtten et al. 1998; Wassman and VanEtten 1996), accumulation of CDCs as achieved in this study has never been reported. To our knowledge, this report is also the first on artificial accumulation of fungal supernumerary chromosomes.

RESULTS

Protoplast fusion.

Protoplast fusion experiments were performed to obtain fusion products containing multiple copies of *PDA1* CDC. Our concern was to achieve accumulation of *PDA1* CDC in the same genetic background as that of either of the fusion parents. For this purpose, we fused two transformants, Tr18.5 and Tr17.2, that were derived from a single strain, 77-13-3, but marked with different selectable genes. Tr18.5, a gift from H. D. VanEtten, has hygromycin B resistance genes (*hph*) inserted into the *PDA1* CDC by homologous recombination (Fig. 1B) (Wassman and VanEtten 1996). We obtained Tr17.2 during the present study by

transforming 77-13-7 with a geneticin resistance gene, neomycin phosphotransferase gene (*nptII*) and selecting Tr17.2 out of 62 transformants for its high level of resistance and colony features similar to 77-13-7. The PFGE Southern hybridization analysis indicated that at least one copy of *nptII* was incorporated into a large chromosome in Tr17.2 by ectopic integration.

Prior to the fusion experiment, the regeneration frequency of the protoplasts on the regeneration medium without selective agents was measured. The frequencies ranged from 5 to 52%, indicating that protoplasts prepared with our protocol were of satisfactory quality for the fusion experiments. Four fusion experiments, including one polyethylene glycol (PEG)-mediated and three electrofusion, were conducted. In the PEG-mediated fusion, 8×10^6 protoplasts of each parent were mixed and treated with PEG, yielding approximately 120 colonies on the regeneration medium containing both hygromycin B and geneticin. In the electrofusion, a mixture of equal amounts of protoplasts (5 to 6×10^6) from each parent was subjected to electric pulses to yield 9, 6 and 22 colonies on the selective medium in each of the three experiments, respectively. After confirming drug resistance and single spore isolation (details below), 26 isolates (one from PEG-mediated fusion, 25 from electrofusion) were obtained (hereinafter, referred to as fusion product and numbered and labeled with prefix "F"). These fusion products were similar to each other as well as to the par-

Fig. 1. Polymerase chain reaction (PCR) amplification of three genes in the fusion products and parent strains of *Nectria haematococca*. **A**, Positioning of specific primers for internal region of *PDA1*. **B**, Primer set for hygromycin resistance genes (*hph*) inserted in the disrupted *PDA1*. "P" denotes the promoter and "T" the transcription terminator of disruptant vector. Not all the restriction sites are shown. **C**, Gel electrophoresis of the PCR-amplified fragment of *PDA1* using primer sets shown in A. **D**, Gel electrophoresis of the PCR-amplified fragment of *hph* and neomycin phosphotransferase gene (*nptII*) using primer sets shown in B. 77-13-7 = wild-type parent of Tr17.2 and Tr18.5, geneticin- and hygromycin B-resistant parents, respectively; F1 to F23 = selected fusion products containing two *PDA1* conditionally dispensable chromosomes.

Fig. 2. Drug sensitivity of fusion products compared with their parents: 77-13-7, wild-type parent of Tr17.2 and Tr18.5, geneticin- and hygromycin B-resistant parents, respectively; F1 and F2, selected fusion products containing two *PDA1* conditionally dispensable chromosomes.

ents with respect to the level of drug resistance, colonial morphology, and growth rate (Fig. 2; detailed data not shown).

In polymerase chain reaction (PCR) tests, all 26 fusion products had both *nptII* and *hph* (Fig. 1D). In other PCR tests that targeted the intact *PDA1* gene, 21 of 26 fusion products were shown to have this intact gene, and the other 5 did not. Altogether, these analyses indicated that all the fusion products were derived from nuclear fusion between the two parents, with the suggestion that most fusion products (approximately 80%) harbored two *PDA1* CDCs, one from Tr17.2 and the other from Tr18.5, while the minority (approximately 20%) harbored only one type of *PDA1* CDC derived from Tr18.5. For further analysis, seven fusion products, including F1 from the PEG fusion and F4, F5, F7, F9, F19, and F23 from the electrofusion, were selected. F5 was estimated to have one *PDA1* CDC and the others to have two *PDA1* CDCs.

Electrophoretic karyotyping.

Strictly speaking, the PCR analysis confirmed the presence of the marker gene but not of the chromosome itself. To obtain direct evidence for the presence of *PDA1* CDC, we used PFGE analysis.

First, a 1.6-Mb chromosome was separated using short-running conditions, followed by Southern-blotting hybridization with *hph* and intact *PDA1* as probes (Fig. 3). Each probe specifically hybridized to the 1.6-Mb band of either parent to enable distinguishing *PDA1* CDC from the parents. In the fusion products, on the other hand, a 1.6-Mb band hybridized with both probes, indicating that this band comprised the co-migrating *PDA1* CDCs from both parents.

The electrophoretic karyotypes of the three fusion parents then were analyzed using long-running conditions to allow separations in the size range of approximately 0.5 to 6 Mb (Fig. 4A). Although chromosomes larger than approximately 6 Mb were difficult to discern due to clumping of bands, banding profiles similar to those of the fusion parents were obtained, suggesting that chromosome rearrangements were not extensive in the fusion products. As an exception, a novel 1.1-Mb band immediately below the 1.6-Mb band appeared in F5, probably due to rearrangement of an A chromosome, as shown by cytological analysis.

Cytological analysis.

Although PFGE Southern analysis revealed the co-migration of two types of *PDA1* CDCs on a 1.6-Mb band, the exact num-

ber of the chromosome in this band remained unclear. Therefore, chromosome number in the fusion parents was analyzed cytologically in two ways. That is, all chromosomes, including *PDA1* CDC and other chromosomes, were counted in 4',6-diamidino-2-phenylindole (DAPI)-stained metaphase specimens prepared by the germ tube burst method (GTBM), whereas *PDA1* CDC was selectively counted using fluorescence in situ hybridization (FISH) chromosome painting.

The total chromosomes obtained for more than 10 specimens always fell in the range of 15 to 17 for each fusion product and parent (Fig. 5), and the variation can be interpreted as sample error due to the inherent nature of the GTBM (Taga et al. 1998; Tsuchiya and Taga 2001). With the results of PFGE yielding similar banding profiles for the fusion products and the parents, these counts apparently indicated that they were haploids containing only one set of A chromosomes.

Fig. 4. Electrophoretic karyotyping of fusion products and their parents. **A**, Long-run pulsed-field gel electrophoresis (PFGE). The numbers on the left represent the chromosome sizes of the standards in megabases. The arrowheads mark the higher intensity for conditionally dispensable chromosome (CDC) of the fusion products compared with the other bands in the same lane. **B**, Results of PFGE Southern analysis to find the chromosomal location of the neomycin phosphotransferase gene (*nptII*) in Tr17.2; 77-13-7, the wild parent of Tr17.2, also was applied as a control in both PFGE and genomic Southern analysis. **C**, Genomic Southern hybridization of 77-13-7 and Tr17.2 to find the copy number of for *nptII* integration. Genomic DNA (3 µg) of 77-13-7 and Tr17.2 digested with *HindIII* and after Southern blotting was hybridized with the same probe of *nptII*. Lane 1 = *Saccharomyces cerevisiae* (standard); lane 2 = wild-type strain 77-13-7; lane 3 = geneticin-resistant parent, Tr17.2; lane 4 = hygromycin B-resistant parent, Tr18.5; lanes 5 and 6 show F7 and F9, respectively; lane 7 = F5, a fusion product containing only one CDC plus an extra band of 1.1 Mb.

Fig. 3. Pulsed-field gel electrophoresis (PFGE) Southern analysis in selected fusion products and parents. **A**, Separation of *PDA1*- conditionally dispensable chromosome (CDC) by PFGE short-running conditions. **B**, PFGE Southern analysis of CDC using hygromycin B resistance genes (*hph*) as probe. **C**, Reprobings of the same blot using internal region of *PDA1* as probe. Lane 1 = geneticin-resistant parent, Tr17.2; lane 2 = wild-type strain 77-13-7; lane 3 = hygromycin B-resistant parent, Tr18.5; lanes 4 to 9 are selected fusion products, F1, F4, F7, F9, F19, and F23, respectively, with two CDCs.

Subsequently, the number of *PDA1* CDCs was determined by chromosome painting via FISH using chromosomal DNA from the 1.6-Mb band of Tr18.5 as the probe. Using more than 10 good specimens for each strain, we consistently observed

two CDCs in the nuclei of the fusion products, except for F5 (Fig. 5). Thus, in combination with the results from the PFGE Southern analysis, we concluded that the fusion products, except for F5, had two copies of *PDA1* CDCs, one from Tr17.2

Fig. 5. Cytological chromosome counting and fluorescence in situ hybridization (FISH) chromosome painting of the fusion products and parents. **A** through **D**, 4',6-Diamidino-2-phenylindole-stained mitotic metaphase chromosomes for chromosome counting. **A**, Tr17.2 (17 chromosomes counted); **B**, Tr18.5 (15 chromosomes); **C**, F4 (17 to 18 chromosomes); **D**, F7 (17 chromosomes); and **E**, F23 (16 chromosomes). The variations in chromosome number are caused by the germ-tube burst method itself, and the real difference in chromosome number is not shown. The photographs are representative for each strain. Scale bar = 1 μ m. **E** through **K**, FISH chromosome painting for detecting *PDA1* conditionally dispensable chromosome. DNA from the 1.6-Mb band of Tr18.5 was used as the probe, and hybridization was detected with avidin-FITC (green). PI was used as a counter stain (red). **F**, Tr17.2; **G**, Tr18.5; **H**, F5; **I**, F1; **J**, F4; **K**, F7; **L**, F9; **M**, F19; and **N**, F23. Nuclei show different growth stages from interphase to metaphase of mitosis. Scale bar = 5 μ m. Cytological analysis were performed for the six selected fusion products and their parents.

Fig. 6. Virulence of fusion products and parents on epicotyls of pea seedlings. Lesions were produced by the "test tube" method (VanEtten et al. 1980), recorded and photographed 6 days after inoculation. **A**, HT-1 without a conditionally dispensable chromosome (CDC); **B**, fusion parent, Tr18.5; **C**, fusion parent, Tr17.2 containing with an intact CDC; **D**, **E**, and **F**, fusion products with two CDCs.

and the other from Tr18.5. In F5, only one *PDA1* CDC was detected, which proved that the novel 1.1-Mb in F5 was not the truncated form of *PDA1* CDC, but originated from another chromosome. Interestingly, hybridization signals for *PDA1* CDC were not dispersed throughout the nucleus but, instead, appeared in a more or less compact form, indicating that this chromosome was condensed to some extent even in somatic interphase nuclei.

Stability of disomic *PDA1* CDCs.

The stability of two *PDA1* CDCs in the genome of fusion products was examined after successive subculturing on PDA that was not amended with antibiotics. The fate of chromosomes was traced by PCR analysis for three marker genes: *nptII*, *hph*, and intact *PDA1*. In the first subculture, all three genes were detected in all six fusion products tested. In the second subculture, however, F1 lost the intact *PDA1* gene, suggesting that the loss of the CDC was derived from Tr17.2. The other five fusion products retained marker genes for the two

PDA1 CDCs until the fourth subculture, but F19 and F23 lost the intact *PDA1* gene in the fifth subculture. Consequently, three of six fusion products retained all three marker genes in the single-spored generation after the eighth subculture. Interestingly, all fusion products retained *hph* through the entire stability test, suggesting the selective maintenance of the *PDA1* CDC from Tr18.5 in the genome of fusion products.

Virulence test.

Virulence of the fusion products on the host plant was assessed with a standard assay using pea epicotyls grown in test tubes (VanEtten et al. 1980). Fusion products produced lesions that were longer on average by 54 to 89, 112 to 160, and 37 to 68% than those produced by the parents Tr17.2 and Tr18.5 and original wild-type strain 77-13-7, respectively (Table 1). This difference in the lesion length between the fusion products and their parents was confirmed with a Tukey-Kramer multiple

Fig. 7. Growth assay of fusion products and their parents on a medium containing homoserine as the sole C and N sources. Tr18.5 and Tr17.2 are hygromycin B- and geneticin-resistant fusion parents, respectively. F1, F4, F7, F9, F19, and F23 are fusion products with two *PDA1* conditionally dispensable chromosomes (CDCs), and F5 with a single *PDA1* CDC. Strain HT-1 that lost *PDA1* CDC was included as an additional control. A conidial suspension (1.5 μ l) was used as inoculum, and the photograph was taken 3 days after inoculation.

Fig. 8. Chromosome counting and fluorescence in situ hybridization (FISH) chromosome painting of a fusion product FN16 obtained from PEG-mediated fusion between two nitrate-nonutilizing (*nir*) mutants that contained two *PDA1* conditionally dispensable chromosomes (CDCs). **A**, 4',6-Diamidino-2-phenylindole-stained mitotic metaphase chromosomes prepared by the germ tube burst method. Approximately 15 to 16 chromosomes can be counted. Scale bar = 1 μ m. **B** and **C**, FISH chromosome painting to detect *PDA1* CDC. DNA from the 1.6-Mb band of Tr18.5 was used as the probe, and hybridization was detected with avidin-FITC (green). PI was used as a counter stain (red). Apparently, four *PDA1* CDCs can be counted in a nucleus. Scale bar = 5 μ m.

Table 1. Studying characteristics of phenotypes correlated with the *PDA1* conditionally dispensable chromosome in *Nectria haematococca*

Fungal Strains ^w	Experiment type ^x	Growth on HS ^y		Virulence on pea	
		Experiment 1	Experiment 2	Lesion length (mean $\pm$ SD) ^y	Lesion increase (%) ^z
77-13-7	...	11.6 c	NA	12.8 $\pm$ 5.0 (10) bc	...
Tr17.2	...	11.4 c	13 d	11.4 $\pm$ 3.3 (18) c	...
Tr18.5	...	NA	12.3 e	8.3 $\pm$ 3.6 (19) c	...
F1	PEG fusion	NA	15.5 (19, 26) bc	19.2 $\pm$ 5.3 (13) a	50, 68, 131
F4	Electrofusion	14.9 (28, 30) b	15.5 (19, 26) bc	17.6 $\pm$ 2.4 (18) ab	37, 54, 112
F7	Electrofusion	15.3 (32, 34) b	16 (23, 30) b	19.6 $\pm$ 3.9 (14) a	53, 72, 136
F9	Electrofusion	NA	15.1 (16, 22) c	21.6 $\pm$ 4.2 (18) a	68, 89, 160
F19	Electrofusion	15.3 (32, 34) b	16 (23, 30) b	18.9 $\pm$ 3.0 (16) a	48, 66, 127
F23	Electrofusion	15.8 (36, 38) a	16.8 (29, 36) a	18.7 $\pm$ 3.4 (15) a	46, 64, 125

^y Diameter of the mycelial colony (mm) after 3 days of growth on semisolid medium containing 0.7% L-homoserine (HS) measured in two independent experiments. Each value is the mean of three replications. Percentage of growth increase of the fusion products compared with 77-13-7 and Tr17.2 (experiment 1) or Tr17.2 and Tr18.5 (experiment 2) are shown in parentheses. Tukey-Kramer multiple-comparison test showed significant difference between parents and fusion products. Means followed by different letters are significantly different ($P < 0.05$); NA = data not available.

^w Parents and fusion products.

^x Type of fusion experiment.

^y Values obtained for pathogenicity of fusion products and their parents on pea (mm). The number of recorded seedlings is given in parenthesis. Tukey-Kramer multiple-comparison and Kruskal-Wallis multiple-comparison Z-value test (Dunn's test) showed significant difference between fusion products and parents. Means followed by different letters are significantly different ($P < 0.05$); SD = standard deviation.

^z Increase in lesion length of fusion products when compared with those of 77-13-7, Tr17.2, and Tr18.5, respectively.

comparison and nonparametric Kruskal-Wallis multiple comparison (Dunn's) tests (Table 1). Because of their higher virulence, the fusion products more rapidly girdled the epicotyls compared with parents and eventually caused damping-off of the seedlings (Fig. 6). In this virulence assay, Tr18.5 was less virulent than Tr17.2, probably because *PDA1* was disrupted in this transformant (Wassman and VanEtten 1996), whereas that of Tr17.2 was intact.

Homoserine utilization.

Because *PDA1* CDC contains an essential gene (or genes) for *N. haematococca* to use homoserine (HS) as a sole carbon and nitrogen source (HS medium) (VanEtten et al. 2001), fusion products were analyzed for their growth on HS medium. The average increase in the colony diameter of fusion products compared with Tr17.2, Tr18.5, and 77-13-7 ranged from 16 to 38, 22 to 36, and 28 to 36%, respectively. Statistical analysis using Tukey-Kramer multiple comparison test indicated that differences between the fusion products and parents are significant (Fig. 7; Table 1).

Accumulation of more than three *PDA1* CDCs.

An attempt was made to achieve more than three copies of *PDA1* CDC per genome. Considering the facilities for generating new selectable markers, we used nitrogen-nonutilizing (*nit*) mutation. After producing many *nit* mutants from the two fusion products F9 and F23 that had stably retained two *PDA1* CDCs in the stability test, we chose two *nit* mutants, F9-3 (NitM) and F23-20 (*nit3*), derived from F9 and F23, respectively, based on strong vegetative complementation. They were subjected to PEG-mediated protoplast fusion using 10^6 protoplasts for each parent; more than 55 prototrophic colonies appeared on the minimal medium and were assessed for stability of the prototrophy (discussed below). Finally, one monoconidial isolate, FN16, with stable prototrophy was obtained. After FISH chromosome painting with the same probe as used in the previous analysis, we observed four *PDA1* CDCs (tetrasomic) in each nucleus of FN16 (Fig. 8B and C). In addition, 14 to 18 chromosomes were counted for each of 15 metaphase spreads prepared by GTBM (Fig. 8A), suggesting that FN16 was haploid. In a PCR analysis, all three markers, *nptII*, *hph*, and intact *PDA1*, were detected in FN16 and its *nit*-mutant parents. Altogether, these results indicate that the genome of *N. haematococca* had accumulated four copies of *PDA1* CDC.

DISCUSSION

Using a common genetic background is a prerequisite for comparing the effects of genes or chromosomes on the phenotype of an organism. Here, we took advantage of intrastain protoplast fusion and spontaneous haploidization of fused nuclei to make fusion products with the same haploid genetic background as that of fusion parents. Consequently, the effects of duplication of *PDA1* CDC on quantitative traits such as virulence could be assessed apart from the effects of A chromosomes. Artificial accumulation of two CDCs (one confirmed to be CD and the other presumed to be so) had been attempted previously in *A. alternata* using protoplast fusion between two pathotypes that produced different host-specific toxins (Salamiah et al. 2001b). The study by Salamiah and associates (2001b), however, is not comparable with ours in that the two CDCs in *A. alternata* substantially differed in size and in gene makeup. More importantly, their fusion products seemed to be heterozygous diploids consisting of two genomes, one from each fusion parent.

Our observation on the haploidization of the fused nuclei is consistent with previous reports for homothallic *N. haemato-*

cocca and *F. oxysporum*, in that the diploid stage produced by protoplast fusion was transient (Daboussi and Gerlinger 1992; Molnar et al. 1990; Teunissen et al. 2002). Thus, no agents such as benomyl were needed to induce or accelerate haploidization in these studies, and it is reasonable to speculate that the genome of *N. haematococca* or *Fusarium* spp. is most stable in the haploid condition and that the extra chromosomes causing aneuploidy or polyploidy are eliminated swiftly from the nuclei. In our stability test, on the other hand, half of the fusion products (three of six) retained two *PDA1* CDCs after successive subcultures under nonselective conditions. Furthermore, even four CDCs accumulated in a fusion product made from *nit* mutants with two *PDA1* CDCs. These results suggest that, to some extent, CDCs are free of the genomic constraint, in contrast to A chromosomes; and, in this regard, are similar to B chromosomes that can be found in multiple copies in somatic cells in other eukaryotes (Camacho 2005). Haploidization is a process constituting the parasexual cycle and accompanies genetic recombination due to chromosome reassortment and mitotic crossing-over. In *Fusarium* spp., Teunissen and associates (2002) showed that every protoplast-fusion product between two races with different karyotypes of *F. oxysporum* f. sp. *lycopersici* contained chromosome rearrangements and recombination. Here, on the contrary, we found chromosomal changes in only one fusion product (F5) of seven in which we detected a novel small chromosome that was derived from the A chromosome. This low level of chromosomal change in our fusion products may be because we performed an intrastain fusion.

For analyzing ploidy and copy number of the CDC in the fusion products, we directly counted chromosomes with cytological methods such as GTBM and FISH. In ploidy analysis, the previous studies mostly employed a genuinely genetic approach using markers such as those for auxotrophy and drug resistance (Daboussi and Gerlinger 1992; Molnar et al. 1990; Teunissen et al. 2002) or, in limited cases, microfluorimetrically measuring nuclear DNA content in hyphal cells (Salamiah et al. 2001b; Talbot et al. 1988). These methods, however, have significant limitations in our system. For example, incorporating numerous markers into the fusion parents to cover most of the chromosome complements without affecting important phenotypes (e.g., virulence) is impractical. Also, the range in values for the DNA measurements would be too wide to reliably detect variation in chromosome number instances (Salamiah et al. 2001b; Talbot et al. 1988). Theoretically, cytological observation can overcome such limitations; and, in fact, convincing data showing the haploid state were obtained using the GTBM in this study. Regarding copy number of the CDC, we used FISH chromosome painting, a basic technique that we used in our earlier work to visualize the *MAK1 PDA6-1* CDC (Taga et al. 1999). Before this, the copy number of a particular CDC was rather difficult to determine with common techniques such as PFGE Southern hybridization and ordinary PCR. For example, Miao and associates (1991) reported asci with an unexpected segregation ratio of 4:4 Pda^+/Pda^- in addition to the expected 8:0 Pda^+/Pda^- in a cross between two strains carrying *MAK1 PDA6-1* CDC. The pda^+ ascospores from such unusual asci might have had two copies of the CDCs as a result of a nondisjunction event at meiotic division I, but their analysis with PFGE Southern hybridization did not clarify the copy number. As demonstrated by this study, FISH painting will serve as a powerful tool to analyze chromosome duplication.

We showed that virulence on pea and the HS-utilizing ability of the fusion products both were elevated by the disomy of *PDA1* CDC. The forced deletion of *PDA1* or *MAK1 PDA6-1* CDC from vegetative nuclei has been done successfully with the aid of benomyl treatment, proving that they are essential

for the two traits (VanEtten et al. 1998, 2001). However, the artificial duplication and dosage effect of CDCs as examined in this study have not been reported before. In our results, the dosage effects of chromosome duplication were remarkably high in virulence (Table 1), while somewhat modest in HS-utilizing ability. Provided that the duplicated genes are expressed normally in the fusion products to yield the double amount of gene products, the modest magnitude in the increase in the HS-utilizing ability may be explained in two ways. First, a set of genes on a single *PDA1* CDC might provide a nearly sufficient amount of the products (enzymes) needed for the HS-utilizing metabolic pathway, which is common in many enzymatic reactions involved in cell metabolism. Second, colony diameter is not an adequate index for measuring gene dosage effects. On the contrary, the increase in the virulence of fusion products suggests that the gene products from a single CDC are not enough to overwhelm resistant response of the host plant against the fungus. Presently, we know that several genes in the *PEP* gene cluster, including *PDA1* on the *PDA1* CDC, play important roles in the virulence of *N. haematococca* MPVI on pea (Han et al. 2001). Because only one of the two *PDA1* CDCs in the fusion products has an intact *PDA1* gene, it is tempting to say that the higher virulence in the fusion products was due to the duplication of *PEP* genes except *PDA1*; however, it is premature to conclude so because the other unidentified genes outside the *PEP* cluster on the same chromosome also might be involved in virulence. To address this issue, extensive disruption experiments for the genes on the *PDA1* CDC and a microarray type of gene expression analysis based on information from the genome project (Joint Genome Institute, *N. haematococca*, v.2) will be necessary.

Our success in making fusion products with higher virulence and HS-utilizing ability urges caution concerning the occurrence of similar strains in the fields. In *Fusarium* spp., hyphal anastomosis, which may lead to nuclear fusion and a subsequent parasexual cycle at a certain frequency, is common (Leslie and Summerell, 2006; Molnar et al. 1990). Therefore, a strain might acquire additional copies of *PDA1* or any other CDC in nature by hyphal anastomosis. Once such a strain is generated, it may survive successfully because of its higher fitness for both causing disease and colonizing the plant rhizosphere. Usually, the vegetative incompatibility system of *Fusarium* spp. inhibits heterokaryon formation between strains with different genetic backgrounds (Leslie and Summerell 2006) and, hence, the probability for duplication of the same CDC by intrastrain anastomosis is expected to be higher than that for the accumulation of CDCs from interstrain anastomosis. In addition to hyphal anastomosis, sexual crossing has the potential to cause CDCs to accumulate in *N. haematococca*, as shown by Miao and associates (1991), who found ascospore progenies containing two copies of *MAK1 PDA6-1* CDC (one was in a truncated form) by sexual crossing. However, such sexual crossing is less likely to occur in the field because the sexual stage of heterothallic *N. haematococca* (i.e., perithecia and ascospores) has rarely been found in nature (VanEtten and Kistler 1988). So far, strains with multiple copies of CDCs have not been isolated from field isolates of *N. haematococca*. This lack does not mean such strains do not exist but presumably reflects the technical difficulty or insufficient efforts in detecting duplicated chromosomes in field isolates. Extensive, careful surveys for such field strains may be a necessary precaution for disease management.

In this study, some interesting features of *PDA1* CDC that could not be revealed by ordinary molecular biological approaches were uncovered for the first time by combining protoplast fusion and cytological and molecular biological techniques. It will be interesting to extend the same kind of analysis

to other CDCs in *N. haematococca* to see if the CDCs have common attributes. The approach we took here may also be applicable to other plant-pathogenic fungi that harbor CDCs. We believe that chromosome manipulation, as demonstrated in this study, should be added to the repertoire of research tools for the genetic study of plant-pathogenic fungi.

MATERIALS AND METHODS

Fungal strains.

Except for transformant Tr17.2 and protoplast fusion products, all strains of *N. haematococca* MPVI used in this study were kind gifts from H. D. VanEtten. Strain 77-13-7, a common ancestor of the other strains, is an ascospore progeny obtained from a sexual cross (Kistler and VanEtten 1984a and b) and contains a single copy of *PDA1* CDC in the genome (Han et al. 2001; VanEtten et al. 1998). Tr18.5 was produced by transformation of 77-13-7; the *PDA1* gene on the *PDA1* CDC was disrupted by homologous recombination with hygromycin-B resistance gene (*hph*) cassette (Wassman and VanEtten 1996). HT-1 is a strain that lost the *PDA1* CDC after benomyl treatment of 77-13-4, another meiotic progeny from the parents of 77-13-7 (VanEtten et al. 2001). Tr17.2 was produced in this study by nonhomologous transformation with geneticin resistant gene cassette (described later).

Strains were maintained on potato dextrose agar (PDA) or Spezieller Nährstoffarmer agar (SNA) (Nirenberg 1976) at 4°C for routine usage. For the fusion products, a medium containing both or either of hygromycin B and geneticin was used. For long-term storage, mycelial plugs were suspended in 50% glycerol and kept at -80°C.

Protoplast preparation.

Protoplasts used for transformation, fusion, and PFGE experiments were prepared as described by Taga and associates (1998) with a different combination of enzymes: 25 mg of driselase (Kyowa Hakko, Tokyo), 4 mg of cellulose Onozuka RS (Yakult Pharmaceuticals, Tokyo), and 4 mg of kitalase (Wako Pure Chemicals, Osaka, Japan) per milliliter of 0.8 M MgSO₄ was used in this study. Protoplasts released in the enzyme solution were harvested and washed twice by centrifugation in 0.5 to 0.8 M NaCl. With 1 ml of enzyme solution, 1 to 5 × 10⁷ protoplasts usually were obtained for each strain.

Transformation.

The geneticin-resistant transformant Tr17.2 was produced by transformation of 77-13-7 with a circular plasmid pII99 containing the neomycin phosphotransferase gene (*nptII*) (Namiki et al. 2001). The transformation process was adapted from Wassman and VanEtten (1996) incorporating modifications as follows. After adding PEG solution (25% [wt/vol] PEG 4,000, 50 mM CaCl₂, and 50 mM Tris-HCl, pH 7.5) to a protoplast suspension (1 to 5 × 10⁶), the mixture was incubated at room temperature for 10 min. Candidate transformants that appeared 4 to 7 days after overlaying with selection agar (1% [wt/vol] water agar (WA) containing geneticin [G418 disulfate, Nacalai Tesque, Kyoto, Japan] at 150 µg/ml) were single-spore isolated and tested for geneticin resistance at 50 µg/ml on PDA and for the *nptII* gene by PCR.

Protoplast fusion.

PEG-mediated fusion was done according to Anne and Peberdy (1976). Equal amounts of protoplasts (8 × 10⁶) in 1 ml of STC buffer (1 M sorbitol, 50 mM Tris-HCl, pH 8.0, 50 mM CaCl₂) from each parent were mixed and concentrated by centrifugation in 100 µl of STC. After dropwise addition of 1 ml of PEG solution (25% [wt/vol] PEG 6,000, 50 mM CaCl₂,

pH 7.5, 10 mM Tris-HCl, and 0.05 M glycine), the mixture was incubated at 30°C for 15 min, then washed twice in 1 M sorbitol by centrifugation. Finally, protoplasts were suspended in 1 ml of STC, mixed with 10 ml of regeneration medium (RGM) (1 M sucrose, 0.1% [wt/vol] casein hydrolysate, 0.1% [wt/vol] yeast extract, and 0.8% [wt/vol] agar), and plated onto RGM base medium containing 1.5% agar. Plates were incubated overnight at 25°C in the dark, then overlaid with WA containing geneticin and hygromycin B (Wako Pure Chemicals) at 150 µg/ml each. After 5 to 7 days, fast-growing colonies were transferred to selective PDA media containing geneticin and hygromycin B, both at 40 µg/ml, to check for dual drug resistance. Tentative fusion products were reisolated by single-spore, subjected to PCR and drug sensitivity as explained later.

For electrofusion, modified procedures of Salamiah and associates (2000a) were used. Briefly, 0.4 ml of protoplast suspensions in fusion buffer (1.0 M sucrose and 0.1 mM CaCl₂ · 2H₂O) containing mixed protoplasts from the two parents (5 to 6 × 10⁶ protoplasts for each) was transferred to a fusion chamber (FTC-02, electrode distance 1 mm; Shimazu, Kyoto, Japan). Protoplasts were fused using a somatic hybridizer (M&S Instrument, Osaka, Japan) under 2.0 MHz AC field at 40 V/mm for 60 s to make pearl chains, followed by three to five DC pulses (40-µs duration, 1-s interval) at 400 V/mm. After pulsation, protoplasts were incubated for 20 min in the fusion chamber at 28°C, then mixed with 10 ml of RGM. Regeneration and screening of fusion products were done as described earlier.

PCR.

Template DNA was isolated from 3-day-old mycelia grown in a well of a 24-well microplate containing 1 ml of YPD (0.3% [wt/vol] yeast extract, 1% [wt/vol] Bacto peptone, and 2% [wt/vol] dextrose). The mycelia were ground, dried, and then, suspended in 0.3 ml of PEX extraction buffer (6.25 mM potassium ethyl xanthatogenate, 100 mM Tris-HCl, pH 7.5, 700 mM NaCl, and 10 mM EDTA, pH 8.0), heated at 60°C for 30 min, and centrifuged at 17,000 × *g* to harvest supernatant. Then, DNA was ethanol-precipitated from the supernatant, after which the pellet was dried and dissolved in distilled water for use.

The internal region (657 bp) of *PDA1* was amplified with primer set *pdaF* (5'-GTTCACTGTCACAAGCGAGCC-3') and *pdaR* (5'-AGACCCAGCCCCAATGTTTCAG-3') under these reaction conditions: an initial step of 2 min at 95°C; followed by 30 cycles of 30 s at 94°C, 20 s at 65°C, and 30 s at 72°C; and a final step of 5 min at 72°C. For detecting the *hph* and *nptII*, primer set *hph-F* (5'-GACGTCTGTCGAGAAGTTTC-3') and *hph-R* (5'-GTATTGACCGATTCCTTGCG-3') (Salamiah et al. 2001a) and primers *npF* (5'-ACGAAGAGCATCAGGGGC-3') and *nptR* (5'-GTAAAGCACGAGGAAGCG-3') were used, respectively. Reaction conditions for the two genes were an initial step of 2 min at 95°C; followed by 30 cycles of 30 s at 94°C, 20 s at 57°C, and 30 s at 72°C; and a final step of 5 min at 72°C.

PFGE and Southern hybridization.

Chromosome-sized DNAs were prepared using approximately 10⁸ protoplasts/ml in low melting agarose (Bio-Rad, Hercules, CA, U.S.A.) according to Miao and associates (1991). DNA was separated on 0.8% agarose gel (pulsed-field certified agarose; Bio-Rad) in 0.5× Tris-borate-EDTA buffer (Sambrook et al. 1989) at 10°C with a contour-clamped homogenous electric field electrophoresis apparatus (CHEF DRII; Bio-Rad). Running conditions to separate chromosomes from 0.5 to 6 Mb were 50 V, ramp pulse time 3,600 to 1,800 s for 115 h; 50 V, 1,800 to 1,300 s for 24 h; 60 V, 1,300 to 800 s for 30 h; and 80 V, 800 to 600 s for 30 h. For the separation of only small

chromosomes, the following conditions were applied: 5.4 V/cm, ramped pulse time 120 to 180 s for 26 h.

For Southern hybridization, chromosomal DNAs separated on a PFGE gel were transferred onto a nylon membrane (Hybond N⁺; Amersham Biosciences, Buckinghamshire, U.K.) by the alkaline-transferring method (Sambrook et al. 1989) using a vacuum-blotting apparatus (Atto Corp., Tokyo). The *hph* and *nptII* probes were amplified by PCR and purified with Qiagen MinElute (Tokyo). The internal region of *PDA1* was dually digested with *SalI* and *SphI* enzymes from the *PDA1* plasmid donated by H. D. VanEtten. The restriction fragment was purified from agarose gel with a GeneClean II kit (Bio 101 Inc., Vista, CA, U.S.A.). For probe labeling and hybridization, an ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences, Freiburg, Germany) was used according to the manufacturer's directions. Signals were detected on X-ray film (Fuji Medical X-ray film; Fuji Film, Tokyo).

Cytology.

Specimens of chromosomes and nuclei for cytological work were prepared by the GTBM using the method of Taga and associates (1998). The conidia were produced on M-100 medium (Stevens 1974). Less than 10⁵ conidial suspension in 200 µl of potato dextrose broth was pipetted onto the center of a clean slide enclosed by paper cement and incubated at 25 to 28°C until the germ tube had grown to an appropriate length.

For chromosome counting, DAPI-stained specimens were observed with a fluorescence microscope (Olympus BH2-RFCA [Tokyo] equipped with UG-1 cube) and a ×100 oil-immersion objective (numerical aperture: 1.3). Micrographs were taken with a digital camera (Olympus CAMEDIA C5050).

FISH chromosome painting was carried out as previously described (Taga et al. 1999). For the probe, the 1.6-Mb *PDA1* CDC band of Tr18.5 was excised from a PFGE gel, and DNA was extracted using GeneClean II kit. The extracted DNA was amplified with a REPLI-g kit (Qiagen) and labeled with biotin-14-dATP by nick translation using a BioNick Labeling System (Invitrogen, Carlsbad, CA, U.S.A.). In the hybridization mixture, 4 µg of genomic DNA from strain HT-1 isolated by the cetyltrimethylammonium bromide procedure (Kerényi et al. 1999) was included to suppress cross-hybridization. Avidin-FITC (Boehringer Mannheim, Indianapolis, IN, U.S.A.) and propidium iodide were used for hybridization detection and counterstaining, respectively. Finally, painted chromosomes were observed with an epifluorescence microscope (Nikon E600 [Tokyo], equipped with UA-1A and B-2A cubes) and micrographs were taken with a CCD camera (Olympus DP-70).

Growth assays.

Resistance of strains to geneticin and hygromycin B was assessed on PDA containing each drug at 40 µg/ml. The ability of HS utilization was tested in two independent experiments, each in triplicate, and radial growth of the strains on a semi-solid medium containing 1.5% (wt/vol) Gerlite gellan gum (Sigma Aldrich, St. Louis) and 0.7% (wt/vol) L-homoserine was measured. As for inocula, the same method as for cytology was used to produce fresh conidia. Glucose (1% wt/vol) instead of homoserine was used for control plates. The data were analyzed by parametric statistical programs (NCSS 2007; NCSS, Kaysville, UT, U.S.A.) to compare the radial growth of colonies.

Stability test.

To check the stability of two CDCs, each selective fusion product was successively cultured eight times on 9-cm plates containing PDA medium without antibiotics so that the fourth

and eighth subcultures were reisolated by single-sporing. DNA was extracted from each culture by the PEX method (Nakahara et al. 1999) followed by PCR using specific primers for *nptII*, *hph*, and *PDA1*.

Virulence assay.

Pathogenicity assays in test tubes were conducted by wound inoculating pea epicotyls using *Pisum sativum* as described by VanEtten and associates (1980). Pea plants (cultivar akabana-tsurunashi, Sakata Seeds, Yokohama, Japan) were grown in test tubes containing vermiculite and nutrient solution (Hyponex Japan, Osaka) at 25°C in the dark for 5 days, and then for 2 days under fluorescent lighting in a high-humidity growth chamber. Epicotyls then were wounded with a small needle (21 G; 0.8 by 38 mm) and inoculated with a 3-mm-diameter plug from 2-day-old mycelium grown on the surface of M-100 medium. Inoculated plants then were incubated in a lighted humidity chamber for 6 days. Virulence was determined by measuring the length of lesions 6 days after inoculation on at least two replicates composed of seven to nine plants each. In control plants, epicotyls were wounded and inoculated with only M-100 medium, and they developed little (1 to 2 mm) or no discoloration at the site of the wound. Parametric and non-parametric statistical analyses were used to compare virulence of the strains.

nit mutants.

The *nit* mutant technique was used with two different well-characterized fusion products to generate mutants to use as parents of a new fusion in order to incorporate more CDC in the cell. The procedures were adapted from Correll and associates (1987). Briefly, plugs taken from fresh culture of two fusion products (F9 and F23) that each contained two CDCs were transferred to PDA amended with 3% potassium chlorate. Chlorate-resistant sectors were found after a 10-day incubation at 25°C. When these sectors were grown on minimal medium (MM), the *nit* mutants appeared as expansive, thin, sparse-growing colonies with no aerial mycelium, indicating their inability to metabolize nitrate. Phenotypes of *nit* mutants were identified by growing each on basal medium amended with various sources of nitrogen (Correll et al. 1987). According to the specific mutation in the nitrate assimilation process, the *nit* mutants fell into three classes: *nit1*, *nit3*, and NitM, as described by Correll and associates (1988). Pairing of *nit1/nit3* with NitM mutants resulted in strong complementation in most combinations. PEG fusion of two *nit* mutants that had strong complementation was done using the method of Molnar and associates (1990).

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LITERATURE CITED

- Anne, J., and Peberdy, J. F. 1976. Induced fusion of fungal protoplasts following treatment with polyethylene glycol. *J. Gen. Microbiol.* 92:413-417.
- Camacho, J. P. M. 2005. B chromosomes. Pages 223-286 in: *The Evolution of the Genome*. T. R. Gregory, ed. Academic Press, Burlington, MA, U.S.A.
- Correll, J. C., Klittich, C. J., Jr. and Leslie, J. F. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology* 77:1640-1646.
- Correll, J. C., Gordon, T. R., and McCain, A. H. 1988. Vegetative compatibility and pathogenicity of *Verticillium albo-atrum*. *Phytopathology* 78:1017-1021.
- Covert, S. F. 1998. Supernumerary chromosomes in filamentous fungi. *Curr. Genet.* 33:311-319.
- Covert, S. F., Enkerli, J., Miao, V. P. W., and VanEtten, H. D. 1996. A gene for maackiain detoxification from a dispensable chromosome of *Nectria haematococca*. *Mol. Gen. Genet.* 251:397-406.
- Daboussi, M. J., and Gerlinger, C. 1992. Parasexual cycle and genetic analysis following protoplast fusion in *Nectria haematococca*. *Curr. Genet.* 21:385-392.
- Enkerli, J., Reed, R., Briley, A. Bhatt, G., and Covert, S. F. 2000. Physical map of a CD chromosome in *Nectria haematococca* MPV1 and location of chromosome breakpoints. *Genetics* 155:1083-1094.
- Han, Y., Liu, X., Benny, U., Kistler, H. C., and Van Etten, H. D. 2001. Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen *Nectria haematococca*. *Plant J.* 25:305-314.
- Hatta, R., Ito, K., Hosaki, Y., Tanaka, T., Tanaka, A., Yamamoto, M., Akimitsu, K., and Tsuge, T. 2002. A conditionally dispensable chromosome controls host specific pathogenicity in the fungal plant pathogen *Alternaria alternata*. *Genetics* 161:59-70.
- He, C., Rusu, A. G., Poplawski, A. M., Irwin J. A. G., and Manners, J. M. 1998. Transfer of a supernumerary chromosome between vegetatively incompatible biotypes of the fungus *Colletotrichum gloeosporioides*. *Genetics* 150:1459-1466.
- Johnson, L. J., Johnson, R. D., Akamatsu, H., Salamiah, A., Otani, H., Kohmoto, K., and Kodama, M. 2001. Spontaneous loss of a conditionally dispensable chromosome from the *Alternaria alternata* apple pathotype leads to loss of toxin production and pathogenicity. *Curr. Genet.* 40:65-72.
- Jones, R. N. 1991. B-chromosome drive. *Am. Nat.* 137:430-442.
- Kerényi, Z., Zeller, K., Hornok, L., and Leslie, J.F. 1999. Molecular standardization of mating type terminology in the *Gibberella fujikuroi* species complex. *Appl. Environ. Microbiol.* 65:4071-4076.
- Kim, H. G., Meinhardt, L. W., Benny, U., and Kistler, H.C. 1995. *Nrs1*, a repetitive elements linked to pisatin demethylase genes on a dispensable chromosome of *Nectria haematococca*. *Mol. Plant-Microbe Interact.* 8:524-531.
- Kistler, H. C., and Benny, U. 1992. Autonomously replicating plasmids and chromosome rearrangement during transformation of *Nectria haematococca*. *Gene* 117:81-89.
- Kistler, H. C., and VanEtten, H. D. 1984a. Three non allelic genes for pisatin demethylation in the fungus *Nectria haematococca*. *J. Gen. Microbiol.* 130:2595-2603.
- Kistler, H. C., and VanEtten, H. D. 1984b. Regulation of pisatin demethylation in *Nectria haematococca* and its influence on pisatin tolerance and virulence. *J. Gen. Microbiol.* 130:2605-2613.
- Leslie, J. F., and Summerell, B. A. 2006. *The Fusarium Laboratory Manual*. Blackwell Publishing, Ames, IA, U.S.A.
- Maloney, A. P., and VanEtten, H. D. 1994. A gene from the fungal plant pathogen *Nectria haematococca* that encodes the phytoalexin-detoxifying enzyme pisatin demethylase defines a new cytochrome P450 family. *Mol. Gen. Genet.* 243:506-514.
- Masunaka, A., Ohtani, K., Peever, T. L., Timmer, L. W., Tsuge, T., Yamamoto, M., Yamamoto, H., and Akimitsu, K. 2005. An isolate of *Alternaria alternata* that is pathogenic to both tangerines and rough lemon and produces two host selective toxins, ACT- and ACR-toxins. *Phytopathology* 95:241-247.
- Miao, V. P. W., Covert, S. F., and VanEtten, H. D. 1991. A fungal gene for antibiotic resistance on a dispensable (B) chromosome. *Science* 254:1773-1776.
- Mills, D., and McCluskey, K. 1990. Electrophoretic karyotypes of fungi: The new cytology. *Mol. Plant-Microbe Interact.* 3:351-357.
- Molnar, A., Sulyok, L., and Hornok, L. 1990. Parasexual recombination between vegetatively incompatible strains in *Fusarium oxysporum*. *Mycol. Res.* 94:393-398.
- Nakahara, K., Hataya, T., and Uyeda, I. 1999. A simple, rapid method of nucleic acid extraction without tissue homogenization for detecting viroids by hybridization and RT-PCR. *J. Virol. Methods* 77:47-58.
- Namiki, F., Matsunaga, M., Okuda, M., Inoue, I., Nishi, K., Fujita, Y., and Tsuge, T. 2001. Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. sp. *melonis*. *Mol. Plant-Microbe Interact.* 14:580-584.
- Nirenberg, H. I. 1976. Untersuchungen über die morphologische und biologische differenzierung in der *Fusarium*-Sektion *Liseola*. *Mitt. Biol. Bundesanst. Land. Forstwirtschaft. Berlin-Dahlem* 169:1-117.
- Salamiah, Fukumasa-Nakai, Y., Akamatsu, H., Otani, H., Kohmoto, K., and Kodama, M. 2001a. Genetic analysis of pathogenicity and host-spe-

- cific toxin production of *Alternaria alternata* tomato pathotype by protoplast fusion. J. Gen. Plant Pathol. 67:7-14.
- Salamiah, Akamatsu, H., Fukumasa-Nakai, Y., Otani, H., Kohmoto, K., and Kodama, M. 2001b. Construction and genetic analysis of hybrid strains between apple and tomato pathotypes of *Alternaria alternata* by protoplast fusion. J. Gen. Plant Pathol. 67:97-105.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U. S.A.
- Shiflett, A. M., Enkerli, J., and Covert, S. F. 2002. *Nhr2*, a copia LTR retrotransposon from a CD chromosome in *Nectria haematococca*. Curr. Genet. 41:99-106.
- Stevens, R. B. 1974. Mycology Guidebook. University of Washington Press, Seattle.
- Taga, M., Murata, M., and Saitoh, H. 1998. Comparison of different karyotyping methods in filamentous ascomycetes—a case study of *Nectria haematococca*. Mycol. Res. 102:1355-1364.
- Taga, M., Murata, M., and VanEtten, H. D. 1999. Visualization of a conditionally dispensable (CD) chromosome in the filamentous ascomycete *Nectria haematococca* by fluorescence *in situ* hybridization. Fungal Genet. Biol. 26:169-177.
- Talbot, N. J., Coddington, A., Roberts, I. N., and Oliver, R. P. 1988. Diploid construction by protoplast fusion in *Fulvia fulva* (syn. *Cladosporium fulvum*): Genetic analysis of an imperfect fungal plant pathogen. Curr. Genet. 14:567-572.
- Teunissen, H. A. S. Verkooijen, J., Cornelissen, B. J. C., and Haring, M. A. 2002. Genetic exchange of avirulence determinants and extensive karyotype rearrangement in parasexual recombinants of *Fusarium oxysporum*. Mol. Genet. Genomics 268:298-310.
- Tsuchiya, D., and Taga, M. 2001. Cytological karyotyping of three *Cochliobolus* spp. by the germ tube burst method. Phytopathology 91:354-360.
- VanEtten, H. D., and Kistler, H. C. 1988. *Nectria haematococca* mating population I and VI. In: Advances in Plant Pathology, Vol. 6. Genetics of Plant Pathogenic Fungi. G. S. Sidhu, ed. Academic Press, London.
- VanEtten, H. D., Matthews, P. S., Tegtmeyer, K. J., Dietert, M. F., and Stein, J. I. 1980. The association of pisatin tolerance and demethylation with virulence on pea in *Nectria haematococca*. Physiol. Plant Pathol. 16:257-268.
- VanEtten, H. D., Jorgensen, S., Enkerli, J., and Covert, S. F. 1998. Inducing the loss of conditionally dispensable chromosomes in *Nectria haematococca* during vegetative growth. Curr. Genet. 33:299-303.
- VanEtten, H. D., Straney, D. C., Covert, S. F., and Kistler, H.C. 2001. The genetics of *Nectria haematococca* mating population VI with special emphasis on its conditionally dispensable (CD) chromosomes: A source of habitat specific genes. In: *Fusarium*. Paul E. Nelson Memorial Symposium. B. A. Summerell, J. F. Leslie, D. Backhouse, W. L. Bryden, and L. W. Burgess, eds. American Phytopathological Society Press, St. Paul, MN, U.S.A.
- Wassman, C. C., and VanEtten, H. D. 1996. Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca* on pea. Mol. Plant-Microbe Interact. 9:793-803.
- Zolan, M. E. 1995. Chromosome-length polymorphism in fungi. Microbiol. Rev. 59:686-698.

AUTHOR RECOMMENDED INTERNET RESOURCE

Genome project of *N. haematococca*:
genome.jgi-psf.org/Necha2/Necha2.home.html