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A Homokaryotic Derivative of a *Phanerochaete chrysosporium* Strain and Its Use in Genomic Analysis of Repetitive Elements

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Analysis of complex gene families in the lignin-degrading basidiomycete *Phanerochaete chrysosporium* has been hampered by the dikaryotic nuclear condition. To facilitate genetic investigations in *P. chrysosporium* strain BKM-F-1767, we isolated a homokaryon from regenerated protoplasts. The nuclear condition was established by PCR amplification of five unlinked genes followed by probing with allele-specific oligonucleotides. Under standard nitrogen-limited culture conditions, lignin peroxidase, manganese peroxidase, and glyoxal oxidase activities of the homokaryon were equivalent to those of the parental dikaryon. We used the homokaryon to determine the genomic organization and to assess transcriptional effects of a family of repetitive elements. Previous studies had identified an insertional mutation, *Pce1*, within lignin peroxidase allele *lipI2*. The element resembled nonautonomous class II transposons and was present in multiple copies in strain BKM-F-1767. In the present study, three additional copies of the *Pce1*-like element were cloned and sequenced. The distribution of elements was nonrandom; all localized to the same 3.7-Mb chromosome, as assessed by segregation analysis and Southern blot analysis of the homokaryon. Reverse transcription-PCR (RT-PCR) showed that *Pce1* was not spliced from the *lipI2* transcript in either the homokaryon or the parental dikaryon. However, both strains had equivalent lignin peroxidase activity, suggesting that some *lip* genes may be redundant.

The most efficient lignin-degrading microorganisms are the white-rot basidiomycetes, of which *Phanerochaete chrysosporium* strain BKM-F-1767 has been the most extensively characterized (18, 25). Under appropriate culture conditions, the fungus secretes multiple isozymes of lignin peroxidase (LiP) and manganese peroxidase (MnP) as well as of the cellulase cellobiohydrolase I. These isozymes are encoded by complex families of structurally related genes, although the precise number of genes and the relationships among the sequences have not been determined. Difficulties differentiating allelic variants from closely related genes and the lack of an accepted standardized nomenclature have complicated the issue (14).

Allelism could be experimentally resolved if cloning was routinely performed with cultures originating from single basidiospores, which are the homokaryotic products of meiosis. The haploid homokaryons possess a single nuclear type, in contrast to the two different nuclear types found in dikaryons. Analyses of single-basidiospore cultures have been used to differentiate alleles and to create genetic and physical maps of *P. chrysosporium* (10, 14, 16, 23, 27, 28, 31, 33, 36, 37). However, single-basidiospore strains typically exhibit reduced sporulation, growth rate, and enzyme yields relative to the parental strain (31, 43). Further, chromosome lengths and other aspects of genome organization are not maintained through meiotic recombination, limiting the experimental value of single-basidiospore strains (10, 13, 23, 37, 44). Homokaryons of nonmeiotic origin would greatly simplify analysis of complex gene families without the disadvantages incurred by recombination.

In addition to the intensively studied gene families, *P. chrysosporium* contains at least one family of repetitive elements. A 1,747-bp insertional element designated *Pce1* was detected in LiP gene *lipI2* (15). The element had features common to class II nonautonomous transposons (11, 20), such as inverted terminal repeats, and a putative target site duplication (TA), and it was present in multiple copies in BKM-F-1767 and in other strains of *P. chrysosporium*. However, *Pce1* contains no extended open reading frames and shows no sequence similarity to known transposases. A single 3.7-Mb chromosome was observed on pulsed-field gel Southern blots probed with *Pce1*, suggesting a nonrandom distribution of *Pce1*-like sequences. The number, sequence, insertional context, and genomic organization of the additional *Pce1*-like sequences were not previously investigated.

Pce1 is inserted immediately adjacent to the fourth intron of *lipI2*, suggesting that it may be spliced from the mature transcript. Reverse transcription-PCR (RT-PCR) analysis of dikaryotic strain BKM-F-1767 identified a transcript from the wild-type allele, *lipI1*, but not from *Pce1*-disrupted *lipI2* (15). Thus, in the heterozygous dikaryon, *Pce1* transcriptionally inactivates *lipI2* and is not spliced, as are certain transposons of higher plants (24). However, *lipI2* processing in the absence of *lipI1* (i.e., in a homokaryon containing only the *lipI2* allele) was not previously assessed.

To simplify genetic analyses, we isolated and characterized a homokaryotic derivative of BKM-F-1767. The homokaryon was used to investigate the organization and transcriptional effects of *Pce1*-like elements. Four highly conserved elements were sequenced and mapped to a single chromosome. The homokaryon phenotype was similar to that of the parental dikaryon even though *lipI* was transcriptionally inactivated. The homokaryotic strain will simplify identification of new genes in libraries, enhance the resolution of pulsed-field gels, and aid investigations of chromosome-length polymorphisms.

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MATERIALS AND METHODS

Fungal strains and isolation of homokaryons. *P. chrysosporium* BKM-F-1767 was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis. Single-basidiospore progeny from BKM-F-1767 were described elsewhere (14).

Homokaryotic strains were isolated from regenerated protoplasts of the dikaryotic strain BKM-F-1767. Protoplasts were prepared essentially as described by Brody and Carbon (7), with minor modifications. A petri plate containing 25 ml of YEG medium (containing [per liter] 5 g of yeast extract and 20 g of glucose) or YMPG medium (containing [per liter] 2 g of yeast extract, 10 g of malt extract, 2 g of peptone, 10 g of glucose, 2 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, and 1 mg of thiamine) was inoculated with 10^5 to 10^7 conidiospores and incubated at 37°C without shaking. After 22 h of incubation the medium was removed by aspiration and the mycelia were gently washed with 20 ml of MgOsm buffer (0.5 M $MgSO_4$, 0.05 M maleic acid [pH 5.9]) (3). The mycelium was resuspended in approximately 12 ml of Novozyme 234 (10 mg of MgOsm buffer per ml; Novo Industries, Copenhagen, Denmark) and incubated at 37°C until protoplasts had developed (about 90 min). Protoplasts were filtered through two layers of Miracloth (Calbiochem, San Diego, Calif.), pelleted, and resuspended in approximately 10 ml of 1.2 M sorbitol–10 mM Tris-HCl, pH 7.4. Protoplasts were diluted to approximately 10^3 protoplasts ml^{-1} in MgOsm buffer, and 10- to 100-μl aliquots were plated on FMSS medium (17). Plates were incubated at 37°C until colonies were visible, and then the mycelium was transferred to YMPG plates, which were incubated at 37°C.

Dikaryotic and homokaryotic strains were differentiated by PCR amplification of specific genes. For the initial screening of 90 colonies, genomic DNA was amplified with *lipI*-specific primers flanking the *Pce1* insertion site (15). The PCR products were size fractionated on agarose gels and stained with ethidium bromide. Dikaryons were recognized by bands at 2,236 bp and 479 bp, which correspond to *lipI2* and *lipI1*, respectively. Putative homokaryons were further analyzed by PCR amplification of specific genes followed by Southern blot probing with allele-specific oligonucleotides (14). PCR primers and probes for differentiating alleles of *lipE*, *lipJ*, *lipF*, *lipD*, and *glx* were previously reported (14). The *mnp2* alleles were amplified as described elsewhere (6) and differentiated with oligonucleotides 5′-GCGATCCTGTAATTGAA-3′ and 5′-GCGAT GGTGCAACTGGA-3′. Two genes, *lipE* and *lipJ*, lie at opposite ends of a LiP gene cluster (36), whereas *lipF*, *lipD*, *glx*, and *mnp2* are not linked to the cluster or to each other (14, 36).

Culture conditions and enzyme assays. Nitrogen-limited cultures were grown statically at 39°C as described previously (8, 26) and harvested on day 5. LiP activities were assayed by veratryl alcohol oxidation (39). MnP activities were measured by monitoring the oxidation of 2,6-dimethoxyphenol (Aldrich, Milwaukee, Wis.) at 469 nm (41). Glyoxal oxidase activity was assayed as described elsewhere (21, 22) using methylglyoxal as the oxidase substrate and phenol red as the substrate for horseradish peroxidase in a coupled assay.

Isolation and mapping of *Pce1*-like sequences. *Pce1*-like sequences were cloned from a pWE15-based cosmid library of *P. chrysosporium* (13) by probing with the full-length copy of *Pce1*. In some cases, the Universal Genome Walker kit (Clontech Laboratories, Palo Alto, Calif.) was used to clone adjacent regions (34).

Segregation analysis of the *Pce1*-like sequences was performed on 54 single-basidiospore isolates (14). The flanking regions of each element were unique, and element-specific PCR primer pairs were designed with one primer anchored in the element and the other in the flanking region. The sequences of these primers were as follows: *Pce2* flanking primer, 5′-CGACAGGCTTGAACATG AC-3′; *Pce3* flanking primer, 5′-GAGTGTTGACTGCAGCCTTGC-3′; *Pce4* flanking primer, 5′-CTGCCAGGCAAGCTTCAGAGC-3′; *Pce2* and *Pce4* element-specific primer, 5′-TCAACCCAGGAGCATAG-3′; and *Pce3* element-specific primer, 5′-AGCGCTGCAAGACGGTCTGGGATATTG-3′.

Southern blots were used to verify genetic segregation and to establish the nuclear origin of *Pce1*-like elements. Based on segregation analysis, the genomic DNA of single-basidiospore cultures harboring one, two, or four elements were digested with *Xho*I and probed with nick-translated *Pce1* under conditions of high stringency (15). The number and identity of *Pce1*-like sequences in the homokaryon RP78 were established similarly. DNA of the parental dikaryon BKM-F-1767, which contains all the *Pce1* copies, was included as a control.

Transcription of *lipI2*. RT-PCR was used to identify *lipI* transcripts. Poly(A) RNA was derived from 2-week-old Aspen wood chip cultures, which favor *lipI* transcription (19). RT was primed with oligo(dT), and PCR conditions were as described previously (19, 40). Three sets of primers were designed to amplify different regions of *lipI* cDNA, including one set which flanked the *Pce1* insert of *lipI2* (Fig. 1). Primers were based on coding regions conserved in both alleles and were designated A through F, with sequences as follows: A, 5′-GCGCCTGGT TCGATGTTTTGGATG-3′; B, 5′-CGCCAGGGGTGACGTTGTGCTTCT-3′; C, 5′-TCTATCGCTATCTCTCCCGCT-3′; D, 5′-CGGTTCGGGAACAAGGC CATC-3′; E, 5′-CAGCTCCAGATGGCCTTGTTCC-3′; and F, 5′-CGCGGGC GATGGTGTGG-3′. Control PCR reactions contained cosmids carrying either *lipI1* or *lipI2*. Reactions were run for 35 cycles, as previously reported (37), and the products were size fractionated in agarose gels. Similar studies were also performed using RNA from nitrogen-starved cultures, in which levels of *lipI* transcripts are relatively lower (37).

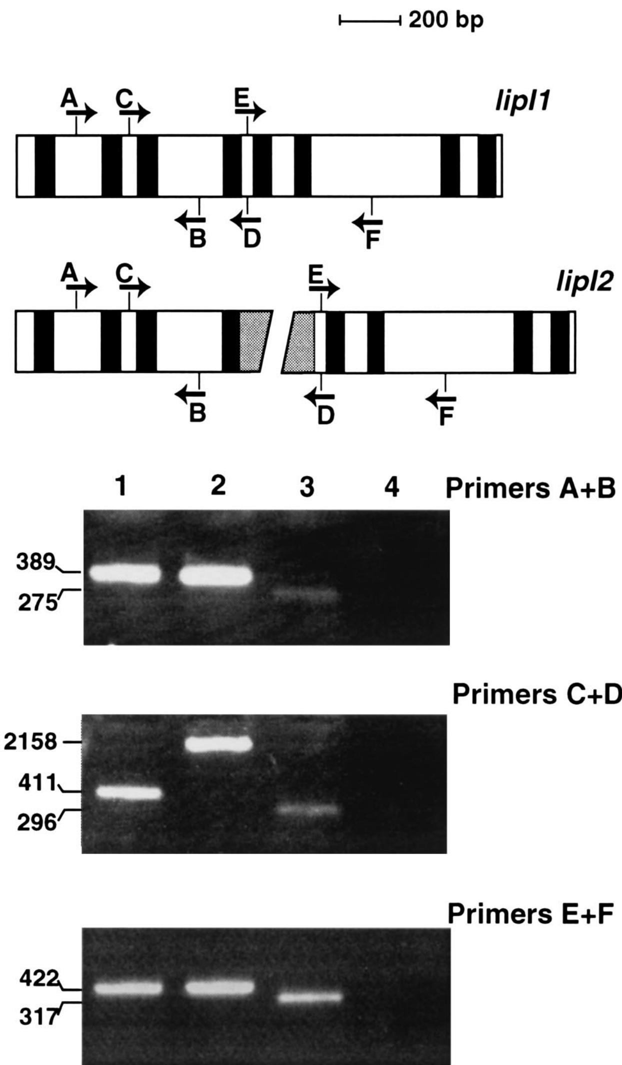

FIG. 1. Analysis of *lipI* transcripts. At the top, a schematic representation of *lipI* alleles shows the locations of introns (black fill), *Pce1* (gray fill), and the primers designated A through F (arrows indicate direction of transcription. Ethidium bromide-stained PCR products size fractionated on agarose gels are shown at the bottom. Lanes 1 and 2 are controls, showing amplification products from cosmid templates carrying *lipI1* and *lipI2*, respectively. RT-PCR products from RNA derived from Aspen wood chip cultures inoculated with BKM-F-1767 and RP-78 are shown in lanes 3 and 4, respectively. No RT-PCR amplification product was obtained from the RP-78 RNA (lane 4). In contrast, BKM-F-1767 cultures contained the expected *lipI* transcript (lane 3). The sizes of the PCR products (in nucleotides) are shown on the left.

Sequence analysis. Nucleotide sequences were determined with the ABI Prism Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, Calif.) with an ABI373 DNA sequencer. Nucleotide and amino acid sequence similarity searches used the BLAST method (5) on the National Center for Biotechnology Information databases.

Nucleotide sequence accession numbers. The elements designated *Pce2*, *Pce3*, and *Pce4*, together with a >900-bp flanking region for each, were assigned GenBank accession numbers AF134289, AF134290, and AF134291, respectively.

RESULTS

Isolation and characterization of homokaryon RP-78. Homokaryotic derivatives of BKM-F-1767 were isolated from regenerated protoplasts, a strategy successfully used for other basidiomycetes (1, 12, 29, 35). In contrast to these previous

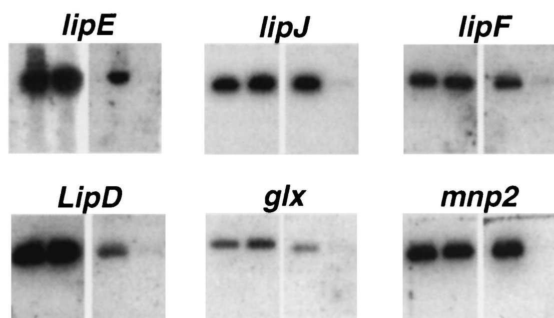

FIG. 2. Southern blot analysis of six PCR amplified genes, demonstrating the homokaryotic nature of RP-78. For each gene, two identical gels were run, with BKM-F-1767 PCR products in the left lanes and RP-78 products in the right. Each gel was then blotted to Nytran and probed with an allele-specific oligonucleotide. The blots show both alleles present in the dikaryotic strain BKM-F-1767 but only a single allele present in RP-78. PCR product sizes were as follows: *lipE*, 1,535 nucleotides (nt); *lipJ*, 675 nt; *lipF*, 1,480 nt; *lipD*, 440 nt; *glx*, 680 nt; and *mnp2*, 1,509 nt.

studies, homokaryons and dikaryons cannot be simply differentiated because *P. chrysosporium* hyphae lack clamp connections (9) and the mating system is not well defined (4, 38).

We identified homokaryons through PCR amplification and allele-specific hybridization techniques. PCR amplification of the dimorphic *lipI* alleles (*lipI2* is disrupted by *Pce1* and is therefore 1,747 bp larger than *lipI1*) was used to distinguish between homokaryons and dikaryons. On this basis, approximately 50% of the 90 regenerated protoplasts were judged dikaryotic and discarded (data not shown). Allele-specific probing of five unlinked genes confirmed that the remaining isolates were all homokaryotic but of the same nuclear type. The existence of a deleterious or lethal allele(s) would most easily explain our inability to recover the second nuclear type.

One homokaryotic isolate, RP-78, was characterized further. In addition to *lipI*, the precise alleles of five unlinked genes were determined using allele-specific oligonucleotide probes (Fig. 2). Under standard N-limited culture conditions, MnP, LiP, and glyoxal oxidase activities of RP-78 were similar to those of the parental strain, BKM-F-1767 (Table 1). On YMPG agar, RP-78 was slower growing but produced more asexual spores than the parent (Table 1).

Genomic organization of the *Pce1* family. We cloned three additional *Pce1*-like sequences designated Pce2, Pce3, and Pce4. Analysis of flanking regions revealed no extended open reading frames and no significant similarity to database sequences. Surprisingly, the three new elements had at least 98% nucleotide identity with *Pce1*. The inverted terminal repeats were identical among the four *Pce1* sequences, but they were imperfect, with 25 of 32 bases conserved in *Pce2*, *Pce3*, and *Pce4*. In short, all four sequences appeared to be the same nonautonomous class II element.

Pce1, *Pce2*, and *Pce3* were linked (Fig. 3), but except for *Pce1*, none were closely linked to genes involved in lignocellulose degradation. *Pce4* was not linked to the other three elements. Because the nucleotide sequences are highly conserved, all would be expected to strongly cross-hybridize under moderate stringency. Thus, the pattern of a previously published pulsed-field gel blot showing hybridization solely to a 3.7-Mb band (15) can be attributed to localization of all *Pce1*-like sequences to a single sister chromosome. Further confirming these results, Southern blots of *Xho*I-digested genomic DNA from RP-78 showed the presence of all four *Pce1*-like elements (data not shown).

Transcription of *lipI2*. Under conditions favoring *lipI* expression (19), RT-PCR showed the presence of *lipI* transcripts in the dikaryotic parent BKM-F-1767 but not in RP-78 (Fig. 1). Conceivably, a *Pce1*-containing transcript might not be efficiently reverse transcribed and PCR amplified. To minimize this possibility, three sets of primers were used, including two which did not flank the insertion (results for primers A plus B and E plus F are shown in Fig. 1). The identity of the *lipI* cDNAs (Fig. 1, lane 3) was confirmed by direct sequencing, and identical results were obtained when RP-78 was grown in nitrogen-starved defined medium (data not shown).

TABLE 1. Enzyme activities and growth characteristics of dikaryotic (BKM-F-1767) and homokaryotic (RP-78) strains

Enzyme activity or growth characteristic	Result[d] for:	
	BKM-F-1767	RP-78
LiP activity[a]	20 ± 9.4	37 ± 6.7
MnP activity[a]	220 ± 36	170 ± 29
Glox activity[a]	6.5 ± 1.6	7.6 ± 3.4
Radial growth (mm)[b]	12 ± 0.6	10 ± 0
Spore production[c] (spores/ml)	$3.5 \times 10^7 \pm 0.6 \times 10^7$	$1.7 \times 10^8 \pm 0.6 \times 10^8$

[a] Enzyme activities are given in nanomoles per minute per milliliter. Glox, glyoxal oxidase.
[b] Radial growth was measured on centrally inoculated YMPG plates after 24 h at 37°C.
[c] Harvested from YMPG plates after 2 weeks at 37°C.
[d] Values are means ± standard deviations.

DISCUSSION

Protoplast-derived homokaryons, termed "protoclones" by Sonnenberg et al. (35), have been identified by other means from other basidiomycetes (1, 12, 29, 35). The nucleus of *P. chrysosporium* protoclone RP-78 is genetically identical to a single nucleus of the dikaryotic strain BKM-F-1767, from which it was derived. RP-78 is genetically distinct from single-basidiospore cultures, which are also homokaryotic but have undergone meiotic recombination. The use of RP-78 should facilitate gene cloning, mutagenesis studies, and additional studies on the genetic mechanisms controlling variation in ligninolytic activity. For example, identifying new members of gene families, either from conventional libraries or by PCR amplification, will be greatly simplified because questions of allelism can be excluded. Pulsed-field gel analysis using RP-78 could avoid dimorphic sister chromosomes (10, 13, 23, 37) and thereby result in improved band resolution.

RP-78 is similar to BKM-F-1767 in its ligninolytic enzyme activity (Table 1) and biomechanical pulping performance (2; D. Dietrich, J. Gaskell, M. Akhtar, R. Blanchette, and D. Cullen, unpublished data). Interestingly, RP-78 LiP activity is approximately the same as that of BKM-F-1767 although it lacks a transcriptionally active *lipI* allele. This observation suggests that LiP activity is influenced by multiple factors and supports the hypothesis that LiP genes are redundant.

Previous Southern blot analysis of BKM-F-1767 suggested multiple *Pce1*-like sequences, and a strong signal was assigned to a single chromosome band of 3.7 Mb (15). Eight *lip* genes also reside on this chromosome and its dimorphic homologue of 3.5 Mb (36). Because of the *Pce* family's unusual distribution, the prospect of isolating an active transposon, and the possibility that other functional genes might be disrupted, three additional *Pce1*-like elements were cloned, sequenced, and mapped.

All four members of the *Pce* family were over 98% identical

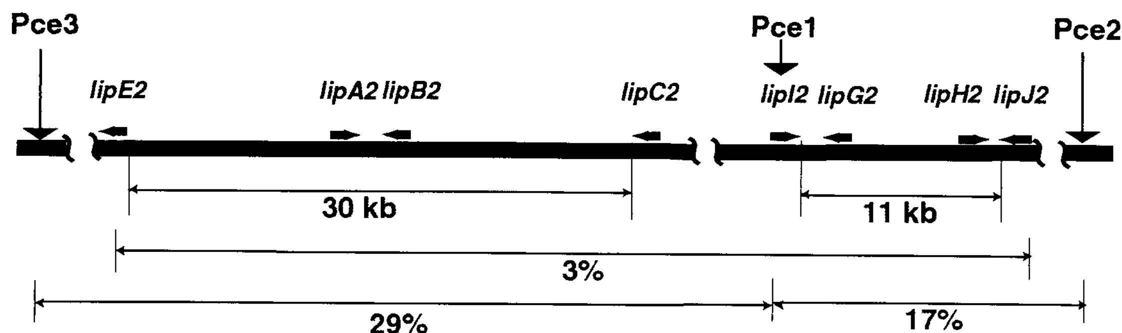

FIG. 3. Genetic linkage of *Pce1*, *Pce2*, and *Pce3* and their relationship to the LiP gene cluster. Linkage was based on analyses of 54 single-basidiospore cultures. Vertical arrows indicate positions of elements. Thickened horizontal arrows show transcriptional orientation of LiP genes (36). *Pce4* segregated independently.

to each other, an unexpected result because none have extended open reading frames (the largest being 155 amino acids). The observed sequence conservation suggests these elements have arisen relatively recently. The *Pce* family may represent the inactive remnants of a transposable element. Approximately 15 copies of the 437-bp element, *vader*, are present in the *Aspergillus niger* genome, while their progenitor, *Tan1*, a 2.3-kb active transposon, is present as a single copy (30). Although no active progenitor of *Pce* was detected in *P. chrysosporium*, the possibility of some biological activity for the *Pce* sequences cannot be excluded.

Segregation analysis showed that *Pce1*, *Pce2*, and *Pce3* were linked. While *Pce4* was localized to the same chromosome, the distance was such that it appeared unlinked. Formally, the possibility of comigrating 3.7-Mb chromosomes (one with *Pce4* and the other with *Pce1*, *Pce2*, and *Pce3*) cannot be dismissed. However, this seems unlikely because none of the more than 25 genes that have been genetically and physically mapped to date (14; S. Alsop, B. Janse, J. Gaskell, A. Vanden Wymelenberg, M. Vallim, and D. Cullen, unpublished data) reside on nonhomologous 3.7-Mb chromosomes. Moreover, *Pce4* clearly cosegregates in the homokaryon RP-78.

The nonrandom distribution of eukaryotic repetitive sequences has been explained by a variety of mechanisms, including site-specific insertions and unequal crossover events between repetitive elements (32, 42). Site-specific insertion, either by specific sequences or by recognition of particular physical characteristics of certain regions of the genome, is not compatible with the available data for the *Pce* family. Over 900 bp flanking each *Pce* insertion site were sequenced, and no obvious similarity was detected.

Unequal crossover between repetitive sequences may be the simplest explanation for the unusual organization of the *Pce* family. Repetitive sequences (such as transposable elements or highly similar gene families) may function as small regions of homology for recombination to occur (44). Unequal crossover between sister chromosomes would generate a duplication in one and a deletion in the other. Thus, mechanisms that may enrich the *Pce* family on a single sister chromosome may also contribute to the observed chromosome length dimorphism. Arguing against this hypothesis, however, the regions surrounding insertion sites for all members of the *Pce* family appear to be conserved on both homologues (data not shown). No duplications or deletions were detected on either homologue. Nevertheless, the *Pce1* family directly contributes to the observed length dimorphism, albeit a small proportion (~8 of ~200 kb). Conceivably, other repetitive sequences may be distributed on the 3.7-Mb homologue.

In conclusion, homokaryon RP-78 was isolated from regenerated protoplasts and shown to be phenotypically similar to its dikaryotic parent, BKM-F-1767. Both RP-78 and BKM-F-1767 contain four copies of the nonautonomous class II transposon *Pce*, all of which mapped to a 3.7-Mb chromosome. The *lipI2* gene of RP-78 has been transcriptionally inactivated by *Pce1* insertion but retains LiP activity equivalent to that of the dikaryon, suggesting that some *lip* genes are redundant. We anticipate that RP-78 will be useful for a variety of genetic analyses, especially differentiating new members of complex gene families.

ACKNOWLEDGMENT

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