Report

Current Biology

Expansion of Signal Transduction Pathways in Fungi by Extensive Genome Duplication

Highlights

- We sequenced the genomes of fungi *Mucor circinelloides* and *Phycomyces blakesleeanus*
- Extensive genomic duplications increased the number of genes for signal transduction
- After duplication, genes specialized their transcriptional regulation by light
- Genome duplications provided new components to improve sensory perception in fungi

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In Brief

Fungi use light and other signals to regulate development, but the mechanisms and origin are largely unknown. Corrochano et al. have found that a group of fungi has acquired new components for signal transduction pathways after extensive genomic duplication. The expanded gene repertoire allows an enhanced perception of environmental signals.

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Report

Expansion of Signal Transduction Pathways in Fungi by Extensive Genome Duplication

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SUMMARY

Plants and fungi use light and other signals to regulate development, growth, and metabolism. The fruiting bodies of the fungus *Phycomyces blakesleeanus* are single cells that react to environmental cues, including light, but the mechanisms are largely unknown [1]. The related fungus *Mucor circinelloides* is an opportunistic human pathogen that changes its mode of growth upon receipt of signals from the environment to facilitate pathogenesis [2]. Understanding how these organisms respond to environmental cues should provide insights into the mechanisms of sensory perception and signal transduction by a single eukaryotic cell, and their role in pathogenesis. We sequenced the genomes of *P. blakesleeanus* and *M. circinelloides* and show that they have been shaped by an extensive genome duplication or, most likely, a whole-genome duplication (WGD), which is rarely observed in fungi [3–6]. We show that the genome duplication has expanded gene families, including those involved in signal transduction, and that duplicated genes have specialized, as evidenced by differences in their regulation by light. The transcriptional response to light varies with the developmental stage and is still observed in a photoreceptor mutant of *P. blakesleeanus*. A phototropic mutant of *P. blakesleeanus* with a heterozygous mutation in the photoreceptor gene *madA* demonstrates that photosensor dosage is important for the magnitude of signal transduction. We conclude that the genome duplication provided the means to improve signal transduction for enhanced perception of environmental signals. Our results will help to understand the role of genome dynamics in the evolution of sensory perception in eukaryotes.

RESULTS AND DISCUSSION

Genome Duplications in the Evolution of the Mucoromycotina Fungi

Gene duplication has expanded the number of genes for photoreception in *P. blakesleeanus* and *M. circinelloides* (Figures 1A and 1B) [7–9], and we hypothesize that gene duplications and specialization may have provided new proteins to expand their sensory repertoire. We thus sequenced the 53.9-Mb *P. blakesleeanus* and the 36.6-Mb *M. circinelloides* genomes,

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and their respective mtDNAs (Supplemental Experimental Procedures; Tables S1A–S1D; Figure S1). The increase in genome size in *P. blakesleeanus* is, in part, due to repetitive DNA, including transcribed transposable elements (Tables S1E–S1G). 16,528 (*P. blakesleeanus*) and 11,719 (*M. circinelloides*) protein-coding genes were annotated and compared to proteins from other fungi (Figure 1C). Comparison of the two genomes with that of *Rhizopus delemar* and other fungi suggests that a whole-genome duplication (WGD) occurred early in the Mucoromycotina lineage. The fungal kingdom contains the subkingdom Dikarya and a number of early divergent lineages including the Mucoromycotina with *P. blakesleeanus*, *M. circinelloides*, and *R. delemar* [10]. Two WGDs have been reported in fungi: in the Saccharomycotina, a lineage of the Dikarya [4–6], and in *R. delemar* [3].

Ancient WGDs are difficult to detect because gene loss and rearrangements result in the absence of regions of synteny. However, genomes from Mucoromycotina species have more members per gene family than genomes from Dikarya fungi (2.9–3.6 versus 1.6–2.2) and a large fraction of gene families with more members than average (50%–68% versus 6.9%–22% for each Dikarya species) (Supplemental Experimental Procedures; Table S2A). Moreover, Mucoromycotina genomes have more duplicated regions than other fungal genomes, with four to 13 genes on average (Tables S2B and S2C). We confirmed the presence of duplicated regions after WGDs in the genomes of *S. cerevisiae* and *R. delemar* as expected, and also large amounts of duplicated DNA in several Dikarya fungi (Tables S2B and S2C). The *Puccinia graminis* and *Laccaria bicolor* genomes have expanded lineage-specific gene families proposed to be involved in pathogenesis and symbiosis [11, 12]. These duplicated regions contain large fractions of lineage-specific genes (Table S2C) supporting the proposal that they arose after species-specific segmental duplications. Additional WGD signatures can be observed in families of three genes from genome pairs. These types of duplicates are more frequent in the genomes of Mucoromycotina species than in non-Mucoromycotina species, suggesting that the former harbors traces of past WGDs (Table S2D).

To gain further insight into past genome expansions in the Mucoromycotina, we reconstructed the complete collection of evolutionary histories (i.e., the phylome) for genes within Mucoromycotina fungi with 13 other fungal genomes. The gene trees were analyzed to detect and date duplication events [13] (Figure 1C). This method has been used to characterize the WGD that took place in the *S. cerevisiae* lineage [14]. In addition to the WGD described in *R. delemar* (0.43 duplications per gene), we detected a larger duplication peak (0.70–0.96 duplications per gene) in the lineage preceding the Mucoromycotina species consistent with a WGD preceding the diversification of this lineage. This early WGD explains gene duplications in the oxidative phosphorylation complex in Mucorales [15] and segmental duplications in *Lichtheimia corymbifera* where previously a species-specific WGD had been rejected [16].

The best explanation for our observations is a WGD predating the diversification of the Mucorales followed by a WGD in the *R. delemar* lineage and, subsequently, rampant gene loss as observed in yeast [5]. The alternative explanation (lineage-specific gene duplications) is less parsimonious. Although segmental duplications can create paralogous regions with shared synteny, we consider it unlikely that numerous such events affecting large regions of the genome would have coincided in time or affected multiple lineages in parallel.

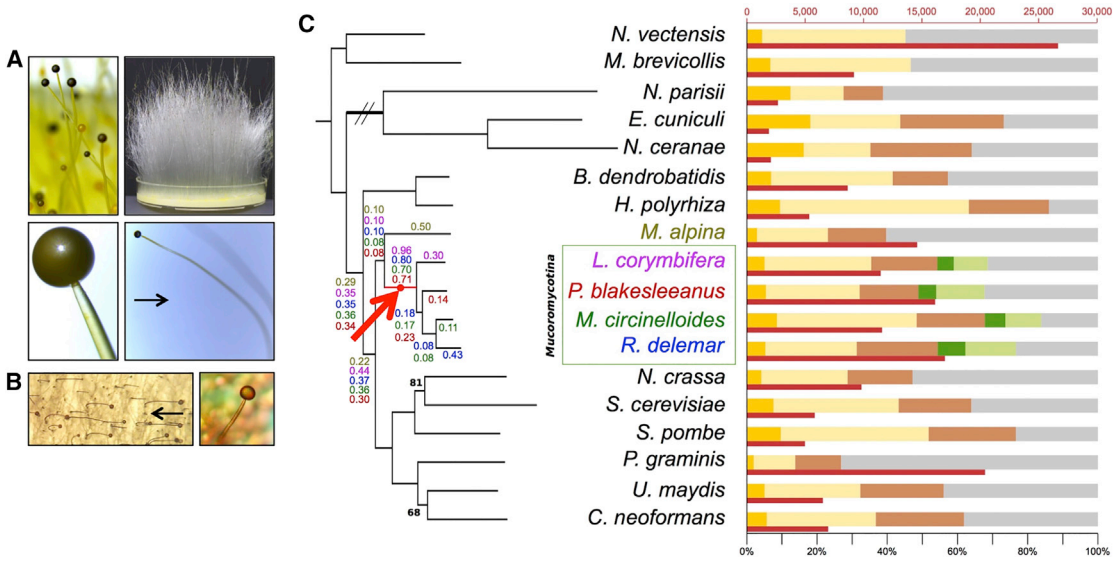

Figure 1. Sensory Perception and Genome Duplication in the Mucoromycotina

(A) The fruiting bodies, sporangiophores, of *Phycomyces blakesleeanus* grow out of the mycelium and reach several centimeters in length. The speed and direction of growth is controlled by signals from the environment including light, gravity, touch, wind, and the presence of nearby objects. The ball at the top of each fruiting body is the sporangium with spores. The direction of light is indicated by an arrow.

(B) The sporangiophores of *Mucor circinelloides* are small (about 5 mm) and show phototropism. The direction of light is indicated by an arrow.

(C) Evidence for a WGD in the Mucoromycotina. A fungal evolutionary tree with bootstrap support values lower than 95% indicated in black numbers at the branches. The average duplication per gene in each lineage is shown with a color that indicates the phylome used for the duplication density calculation. The branch where the proposed WGD took place is marked in red with a dot and an arrow. The graph (scale on the bottom) represents the percentage of genes in a given species that belong to one of the following categories: yellow, protein present in all species; light yellow, ancestral proteins that have homologs in the outgroups; brown, Fungi-specific proteins; green, Mucoromycotina-specific proteins, which appear in all four species; light green, Mucoromycotina-specific proteins; gray, species-specific proteins. The red bars (scale on top) represent the total number of proteins encoded in each genome.

See also Figure S1 and Tables S1 and S2.

Expansion of Gene Families in Mucoromycotina Fungi

Duplicated genes in the four Mucoromycotina species contained an abundance of gene ontology (GO) terms for protein kinase activities (GO 4674, GO 4672, GO 4713, GO 4707; p value at least 1×10^{-6}), fructose 2,6-bisphosphate metabolic process (GO 6003; $p = 1.3 \times 10^{-6}$), ATP binding (GO 5524; $p = 1.8 \times 10^{-46}$), and protein transport (GO 15031; $p = 1.6 \times 10^{-17}$), suggesting duplications of genes encoding signaling pathways and transport components (Supplemental Experimental Procedures; Figure 2; Data S1). This is supported by further analysis of the abundance of signal transduction genes. We have limited this analysis to the genomes of *P. blakesleeanus*, *M. circinelloides*, and *R. delemar* as they were the only Mucoromycotina genomes sequenced when we started the project.

Heterotrimeric G protein signaling is central to the life cycle and virulence of fungi [17, 18]. All the gene families encoding the subunits of heterotrimeric G proteins are expanded. The Gα subunit family contains ten, 12, and 12 genes in *P. blakesleeanus*, *M. circinelloides*, and *R. delemar*, respectively, compared to an average of three in the Ascomycota or seven in the Dikarya (Figure 2A). A single Gβ subunit gene has been found in the Ascomycota as well as in the basidiomycetes *Ustilago maydis* and *Cryptococcus neoformans* [19, 20]. In *P. blakesleeanus*, we identified five Gβ genes, in *M. circinelloides* three, and in *R. delemar* four. Similarly, the Mucoromycotina genomes have three or four genes for the Gα subunit compared to an average of one in the Dikaria (Figure 2A). Theoretically, a very large number of G protein heterotrimers could be built from the multiple subunits. An estimate of 21, 21, and ten genes for G-protein-coupled receptors (GPCR) in *P. blakesleeanus*, *M. circinelloides*, and *R. delemar*, respectively (Data S1, sheet 1), suggests moderate expansion compared to ten to 12 genes in Dikarya [21].

The number of other signal transduction genes has increased compared to the Dikarya, including genes for protein kinases, TRAFAC class GTPases, and regulators of GTPases of the Ras superfamily (Data S1, sheets 2–4). The expansion of kinase families is 3- to 4-fold, as there are 63, 70, and 82 CAMK genes in *P. blakesleeanus*, *M. circinelloides*, and *R. delemar*, respectively, compared to 22 and 21 in *Neurospora crassa* and *U. maydis*. Other families show larger expansion, e.g., 11–18 genes for casein kinase 1 in Mucoromycotina compared to two to three in Dikarya (Figure 2A; Data S1, sheet 4).

Some, but not all, families in a given category are expanded; e.g., for photoreception [22], the genes for components of the WC photoreceptor complex (WC-1 and WC-2) are duplicated, but not the cryptochrome gene; genes for casein kinase 1 are duplicated, but not those encoding the sensor histidine kinases (Figure 2A). Genes for calcium or pH sensing show non-uniform duplication: there are multiple calmodulin genes and three calcineurin catalytic subunit genes, but only a single calcineurin regulatory subunit; in the pH pathway there are three to four genes for the PacC transcription factor and two genes for PalA, yet one gene for PalB or PalC as in the ascomycete *Aspergillus nidulans* [23] (Data S1, sheet 1). Cyclin families are expanded, but there is

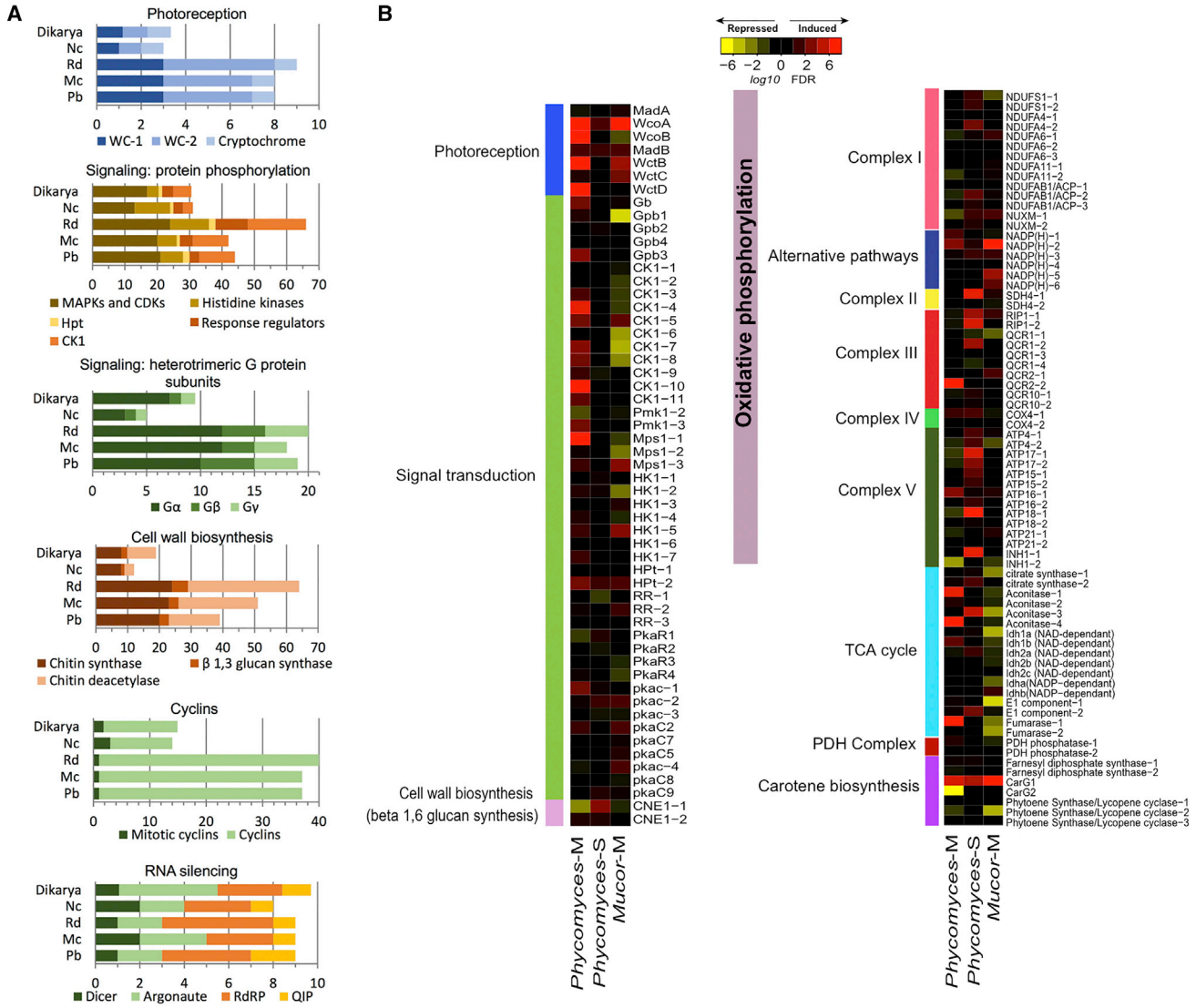

Figure 2. Gene Expansion and Transcriptional Specialization in the Mucoromycotina

(A) Gene abundance in Mucoromycotina, *Neurospora crassa*, and Dikarya fungi. The x axis indicates number of genes, and the bars, from bottom to top, indicate numbers of predicted genes for the three Mucoromycotina (Pb, *P. blakesleeanus*; Mc, *M. circinelloides*; Rd, *R. delemar*), and average number of genes for *N. crassa* (Nc), and Dikarya.

(B) Expression patterns in response to light of duplicated genes in *P. blakesleeanus* and *M. circinelloides*. Differential expression of genes was obtained for two *P. blakesleeanus* stages (mycelium and sporangiophore), and *M. circinelloides* mycelium. The three *wc-1* genes are *madA*, *wcoA*, and *wcoB*, and the four *wc-2* genes are *madB*, *wctB*, *wctC*, and *wctD*. Results are represented with the logarithm base ten of false discovery rate (FDR) (FDR <0.05; fold change >2). M, mycelium; S, sporangiophore.

See also Figure S2 and Data S1.

only a single mitotic cyclin, compared to several in the Dikarya (Figure 2A). Not all gene families have expanded. For example, the genes encoding proteins that participate in genome defense through RNAi have not duplicated (Figure 2A) [24].

The genome duplication has multiplied genes involved in cell wall biosynthesis, in particular, chitin synthases and chitin deacetylases (Figure 2A). These enzymes may have specialized to modulate the growth response of the sporangiophore after environmental stimuli. Functional specialization after gene duplication should have played a key role in *M. circinelloides* and other pathogenic Mucoromycotina fungi. Mutants of the photoreceptor gene *wc-1* of *Fusarium oxysporum* and *C. neoformans* show decreased virulence [25, 26]. *M. circinelloides wc* genes have specialized their sensory role after gene duplication [9], suggesting that some WC proteins may serve as pathogenicity factors (S.T.-M., unpublished data). Calcineurin is a virulence factor in several fungi, including *M. circinelloides* [27]. The number of calcineurin A catalytic subunit genes has increased, with three genes in *M. circinelloides* compared to one in other fungi (Data S1, sheet 1). One of them, *cnaA*, is involved in virulence [27], confirming gene specialization to facilitate pathogenesis after duplication.

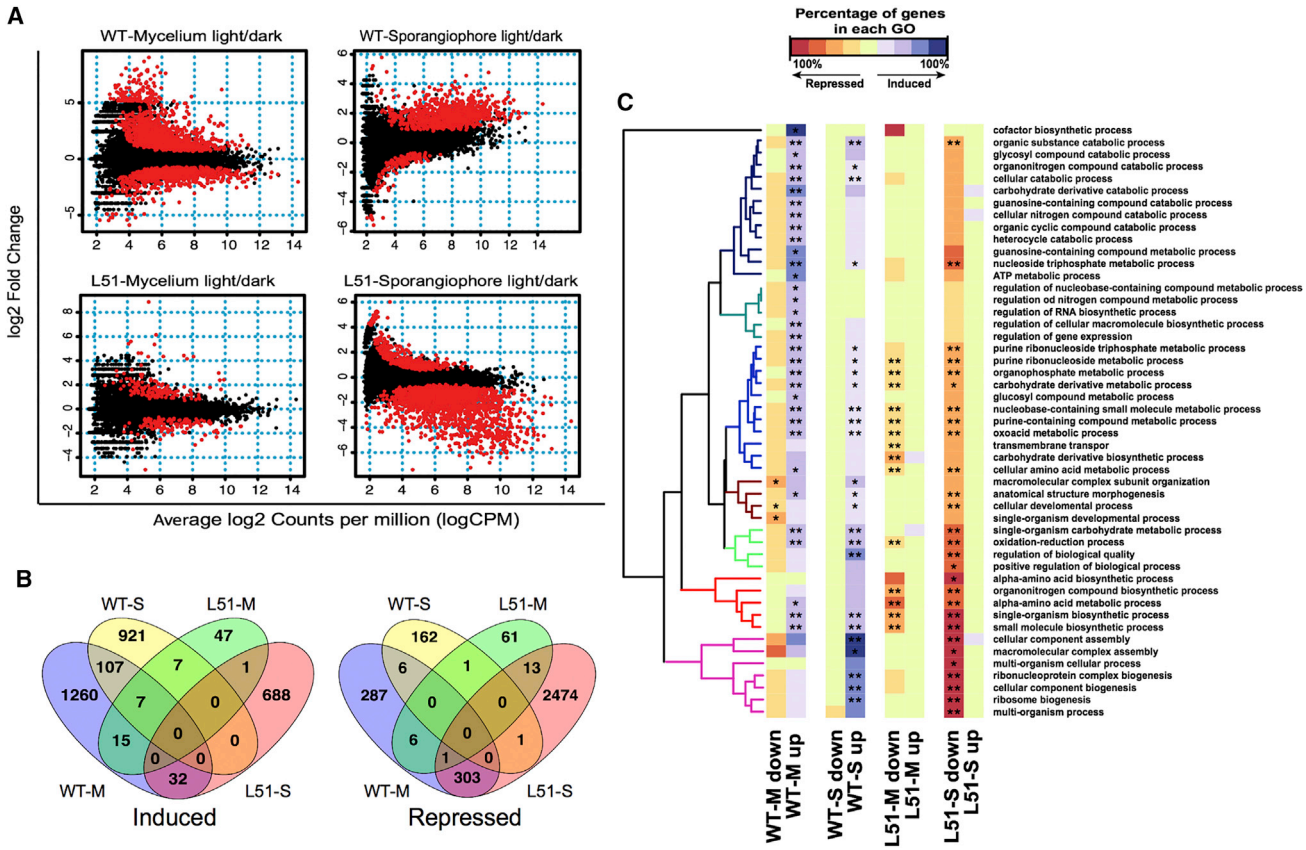

Figure 3. The Influence of Light on Gene Expression in Two Developmental Stages of *P. blakesleeanus*

(A) Differential expression (light/dark) in the mycelium or the sporangiophore of the wild-type and the *madA madB* mutant strain (L51). Differentially expressed genes with FDR $\leq$0.05 are shown in red.

(B) Overlap of genes induced and repressed in the wild-type and mutant using RNA from mycelia (WT-ML and L51-ML) or sporangiophores (WT-SL and L51-SL).

(C) Category enrichment in differentially expressed genes (*FDR <0.05; **FDR <0.01). Each vertical block contains the up- and downregulated categories. Color intensity represents the percentage of genes belonging to each category and includes only GO terms for Biological Processes. Clusters based on this percentage are displayed in different colors in the tree.

See also Data S2.

The expansion of some gene families can be accounted for by a WGD and retention of the resulting paralogs. The large number of chitin deacetylases or Gα subunits genes (Figure 2A; Data S1, sheet 1), however, cannot be explained by a WGD alone, suggesting additional segmental duplications. Having more proteins for signal transduction and cell wall biosynthesis should have helped Mucoromycotina fungi to improve environmental sensing and responses, including the perception of potential hosts for pathogenic fungi. Elucidation of the biological role of duplicated genes will require further characterization.

Duplicated Genes Differ in their Transcriptional Response to Light

To investigate whether duplicated genes have specialized, we asked whether duplicated genes from *P. blakesleeanus* and *M. circinelloides* responded differently to light. The transcriptome of cultures kept in the dark or after exposure to 30 min of blue light (2.3 $\times$ 10^3 J/m^2) indicates specialization (Figure 2B). Most of the genes encoding components of the photoreceptor complex (WCC) or the regulatory subunits of protein kinase A showed a similar expression pattern in *P. blakesleeanus* and *M. circinelloides* with only some genes regulated by light despite being transcriptionally active in both mycelia and sporangiophores (Figure 2B). In addition, genes encoding the photoreceptor WcoB, the Gβ subunits Gpb1 and Gpb3, and the kinases Pkac-1, Mps1-1, CK1-7, and CK1-8, showed opposite responses to light in *P. blakesleeanus* and *M. circinelloides*. Thus, duplicated genes have evolved different patterns of expression in different organisms, as well as between specific tissues in the same organism.

A Refined Transcriptional Response to Light in *P. blakesleeanus*

The expansion in the number of photoreceptors and other signal transduction proteins may have allowed fine-tuning of the response to light, for example, allowing tissue-specific transcriptional responses. We thus characterized the global transcriptional response in *P. blakesleeanus* mycelium and sporangiophores by RNA sequencing (RNA-seq) (Figure 3; Data S2). A total of 2,024 genes were responsive to light in the mycelium (1,421 induced and 603 repressed, about 12% of the protein-coding genes), compared to 1,212 genes in the sporangiophores

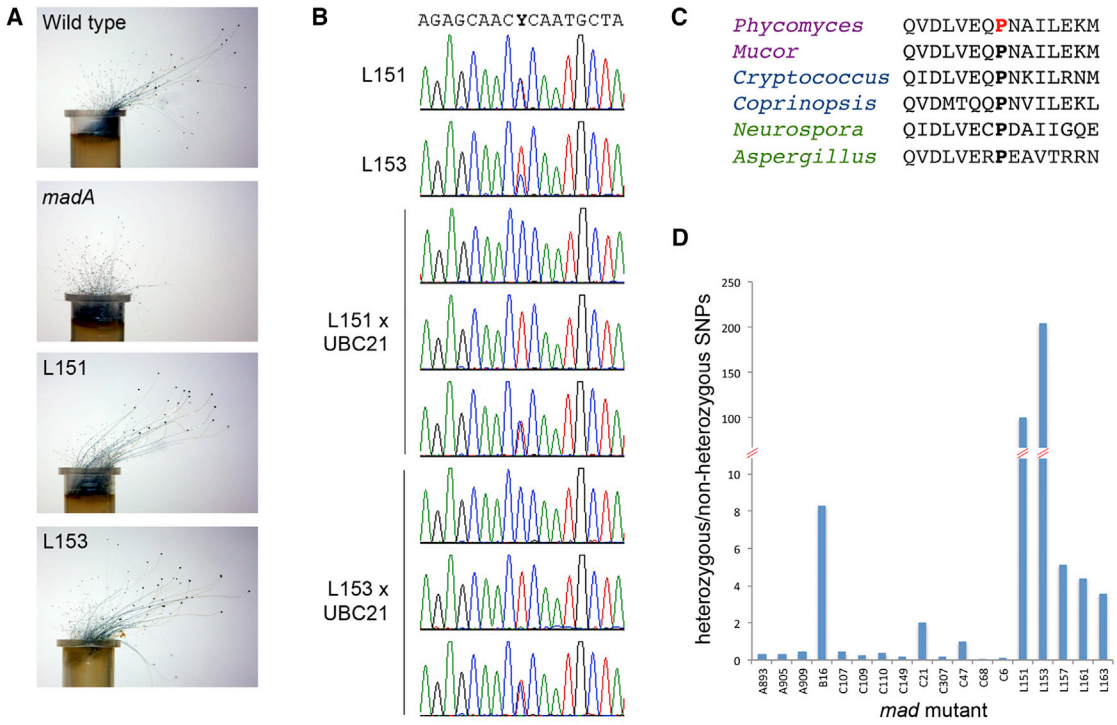

Figure 4. The *P. blakesleeanus madI* Strains Are Heterokaryons of Wild-Type and *madA* Nuclei

(A) Phenotype of wild-type, *madA*, and *madI* strains L151 and L153, with illumination from the right. The average bending response of *madI* strains is lower than in the wild-type strain with the intensity that we used.

(B) DNA sequencing chromatograms of a region of the *madA* gene from the two *madI* strains and progeny from the *madI* mutants crossed to wild-type UBC21. The progeny sequences represent the three different types observed in the strains obtained from crosses.

(C) A segment of the sequence of MadA in different fungi with the conserved proline that is mutated in *madI* strains in bold.

(D) Graph showing the ratio of heterozygous/non-heterozygous SNPs in the genomes of 19 *mad* mutants.

See also Figure S3 and Data S3.

(1,042 induced and 170 repressed). The transcriptional response to light was specific for each developmental stage because only 120 genes were light-regulated in both mycelium and sporangiophores (Figures 3A and 3B). The same analysis on a *madA madB* double mutant (strain L51) that is considered blind [28] detected only 159 light-regulated genes in the mycelium confirming the relevance of the Mad complex (Figure 3A). Surprisingly, the *madA madB* mutant showed a significant response to light in sporangiophores where 3,513 genes were regulated by light (Figure 3A). It is noteworthy that most responsive genes in the *madA madB* mutant sporangiophores were repressed by light (Figure 3A). This suggests the activity of light-dependent repressors in the absence of the Mad complex, as proposed by electrophoretic mobility shift assay (EMSA) experiments that showed the binding of proteins in the dark to a light-regulated promoter in a *madA madB* mutant [29]. Seven gene clusters were enriched in regulatory genes in light-induced mRNAs from the mycelium of the wild-type, and in ribosome biogenesis genes in the sporangiophores (Figure 3C). *P. blakesleeanus* and *M. circinelloides* have 879 and 650 genes encoding transcription factors (TFs), respectively (about 5% of the protein-coding genes), with an abundance of C_2H_2 Zn finger TFs (Figure S2). Light regulates 9% of the *P. blakesleeanus* transcription factor genes (Data S1, sheets 5–7; Data S2, sheets 5 and 6), and we propose that the stage-specific transcriptional response to light relies on the expanded set of photoreceptors and light-dependent transcriptional regulators. Specialization of genes for signal transduction following WGD has been observed in vertebrate vision [30]. Strikingly, in both vertebrates and fungi the expansion of signal transduction genes after WGD has resulted in more elaborate sensory perception.

Reduced Sensitivity to Light in Strains with Wild-Type and *madA* Mutant Nuclei

The photoresponse in the *P. blakesleeanus madA madB* mutant suggested the action of additional photoreceptors. Light perception in *madI* mutants is reduced 10- to 1,000-fold, halfway between wild-type and *madA* mutants, making MadI a candidate for a photoreceptor [31, 32] (Figure 4A). To identify *madI*, we crossed two *madI* strains with a wild-type strain and characterized the phototropism and molecular markers in the progeny [33]. Weak linkage was found for the *madI* mutation and three scaffolds, including the one carrying *madA* (Data S3). We therefore sequenced the genomes of two *madI* mutants (L151 and L153), along with another 17 *mad* mutant strains, and the sequences were scanned across the three scaffolds. We found that the two *madI* strains had an identical and unique mutation in *madA* [8] changing a conserved proline to leucine (Figures 4B and 4C). However, the *madI* strains also contained the wild-type allele, indicating that they were heterozygous for this

gene. To confirm these observations, we sequenced *madA* in 63 *madI* × wild-type progeny: eight only had the mutation in the *madA* gene, 35 were wild-type, and 20 were heterozygotes. The two *madI* mutants had a high number of heterozygous sites across their genomes compared to other strains (Figure 4D). Analysis of all scaffolds in the L151 and L153 genomes showed that the heterozygous SNPs are distributed throughout all chromosomes (Figure S3), suggesting that the two *madI* strains are heterokaryons or diploids, rather than being aneuploid or carrying a segmental duplication. The observation that two of the *madI* strains are heterozygous wild-type/*madA* mutants shows that sensitivity to light is related to the dosage of the MadA photoreceptor.

Our characterization of the genomes of *P. blakesleeanus* and *M. circinelloides*, our comparative fungal genome analysis, and our gene function studies provide new insight into the occurrence and consequences of genome duplications in the evolution of fungi. Expansion and specialization of genes for signal transduction and cell-wall biosynthesis following genome duplication in the Mucoromycotina provided new proteins that have enabled these fungi to refine the way they perceive signals from the environment to regulate their growth and development. Our results provide new genomic tools to unravel the molecular mechanisms of sensory perception in early diverging fungi that will help to understand the evolution of sensory perception in eukaryotes.

ACCESSION NUMBERS

Genome assemblies and annotations have been deposited in DDBJ/EMBL/GenBank: AMYC00000000 (*P. blakesleeanus*) and AMYB00000000 (*M. circinelloides*) and are accessible via the JGI Mycocosm portal (http://jgi.doe.gov/fungi). The mtDNAs have been deposited in DDBJ/EMBL/GenBank: KR809878 (*P. blakesleeanus*) and KR809877 (*M. circinelloides*). Phylomes can be accessed at phylomeDB (http://phylomedb.org) with phylomeIDs 252, 253, 254, 255, and 256. Gene expression data have been deposited in the GEO database: GSE64369 (*P. blakesleeanus*) and GSE58264 (*M. circinelloides*).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables, and three datasets and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.04.038.

AUTHOR CONTRIBUTIONS

A.K., M.M.-H., S.P., A.S., J.M.V.-E., and J.G. contributed equally to this work. L.M.C. coordinated the project. E.L.B., S.B., S.T.-M. and L.M.C. conceived the project. I.V.G. directed all genome-sequencing and analysis efforts at JGI. J.R.-R., V.G.T., A.M.-D., C.S., M.S., E.M.L., F.S.-F., and V.G. provided DNA and RNA samples. A.K., A.S., E.L., J.-F.C., W.S., J.M., J.S., J.G., H.T., and D.B. sequenced, assembled, and annotated the genomes. M.M.-H., A.S., T.G., and I.V.G. did genome comparisons and phylogenomics analysis. M.I.A., J.A., E.P.B., I.B., G.B., L.P.C., D.C., E.C.O., L.M.C., A.D., M.E., A.P.E., V.G., F.G., G.G., J.H., B.H., B.A.H., A.I., E.A.I., B.F.L., J.L.L., S.C.L., W.L., A.T.M., H.R.M., A.M., E.M.-T., J.A.O., R.A.O., M. Olmedo, M. Orejas, L.O.-C., A.G.P., J.R-R., J.R.-H., R.R.-V., C.S., M.S., E.S., F.S.-F., D.S., K.S., V.G.T., N.J.T., M.R.T., S.T.-M., R.P.V., A.W., and J.S.Y. analyzed the genomes. J.M.V.-E., E.M.L., S.L.-G., F.S.-F., R.R.-V., S.T.-M., V.G., L.M.C., and A.H.-E performed the transcriptomic analysis. S.P., S.B., K.M., and A.I. performed the genetic characterization of *madI*. L.M.C., B.A.H., S.T.-M, A.I., A.H.-E., T.G., and I.V.G. wrote the paper with input from all authors.

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