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Systematic Deletion Analysis of Fission Yeast Protein Kinases†

Andrea Bimbó,[1]‡ Yonghui Jia,[2]‡ Siew Lay Poh,[2]§ R. Krishna Murthy Karuturi,[2] Nicole den Elzen,[3] Xu Peng,[2] Liling Zheng,[1] Matthew O'Connell,[3]¶ Edison T. Liu,[2] Mohan K. Balasubramanian,[1,4]* and Jianhua Liu[2,5]*

Temasek Life Sciences Laboratory, 1 Research Link, NUS, Singapore 117604, Singapore,[1] Genome Institute of Singapore, 60 Biopolis Street, Singapore 138672, Singapore,[2] Peter MacCallum Cancer Centre, St. Andrews Place, Melbourne, Vic 3002, Australia,[3] and Department of Biological Sciences[4] and Department of Biochemistry,[5] National University of Singapore, Singapore

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Eukaryotic protein kinases are key molecules mediating signal transduction that play a pivotal role in the regulation of various biological processes, including cell cycle progression, cellular morphogenesis, development, and cellular response to environmental changes. A total of 106 eukaryotic protein kinase catalytic-domain-containing proteins have been found in the entire fission yeast genome, 44% (or 64%) of which possess orthologues (or nearest homologues) in humans, based on sequence similarity within catalytic domains. Systematic deletion analysis of all putative protein kinase-encoding genes have revealed that 17 out of 106 were essential for viability, including three previously uncharacterized putative protein kinases. Although the remaining 89 protein kinase mutants were able to form colonies under optimal growth conditions, 46% of the mutants exhibited hypersensitivity to at least 1 of the 17 different stress factors tested. Phenotypic assessment of these mutants allowed us to arrange kinases into functional groups. Based on the results of this assay, we propose also the existence of four major signaling pathways that are involved in the response to 17 stresses tested. Microarray analysis demonstrated a significant correlation between the expression signature and growth phenotype of kinase mutants tested. Our complete microarray data sets are available at http://giscompute.gis.a-star.edu.sg/~gisljh/kinome.

Eukaryotic cells employ signal transduction pathways to respond to various environmental stress factors and to adapt by reorganizing cellular metabolism, gene expression, cell cycle control, and morphogenesis. Protein kinases are major players in mediating signals to regulate a variety of biological processes in cellular response to environmental stresses. In fission yeast, a number of protein kinases have been shown to be involved in signal transduction, which is rapidly activated upon cells being subjected to various stress factors, for example, the oxidative stress factor H_2O_2 (44). The activation of the signaling pathway Wis4-Wis1-Sty1, reminiscent of the MAK-MKK3/MKK6-p38 mitogen-activated protein (MAP) kinase cascade in mammalian systems (44), leads to the induction of expression of a number of genes whose products are involved in the neutralization and repair of damage caused by H_2O_2 (11, 53). Cells defective for one of the *wis4*, *wis1*, and *sty1* genes exhibit hypersensitivity to H_2O_2, because of failure in mediating signals to reorganize various cellular events, such as metabolism and gene expression (9, 11, 53).

A number of protein kinases involved in mediating signals during cellular response to various environmental changes have been previously characterized. At least three mitogen-activated protein kinase (MAPK) cascades have been analyzed in fission yeast. While the Wis4-Wis1-Sty1 MAPK cascade is involved in the oxidative stress response, the Byr2-Byr1-Spk1 and Mhk1-Pek1-Pkm1 MAPK cascades have been shown to mediate signals in response to pheromones and ion homeostasis and cell wall integrity, respectively (40, 63). Cells containing loss-of-function mutations in these signaling molecules show either defective pheromone responses (40) or sensitivity to cell wall-degrading agents, such as β-glucanases (30, 63). Besides the involvement of MAPK cascades in response to environmental and/or cellular perturbations, a number of protein kinases implicated in response to a variety of stress factors, such as agents that block DNA replication, damage chromosomal DNA, destabilize microtubule structures, and disrupt cell wall structures, have been characterized. For example, Cds1 and Chk1 regulate cell cycle control in response to blocks to DNA replication and/or DNA damage (49). Hhp1 and Hhp2 are involved in DNA damage repair pathways (13, 19). Cells carrying mutations in these kinases display hypersensitivity to agents that block DNA replication and/or damage DNA. Bub1 and Mph1, on the other hand, regulate cell cycle control in response to defective kinetochore capture, and *bub1* and *mph1* mutants show hypersensitivity to microtubule poisons (7, 17). A number of protein kinases have also been reported to regulate cell wall integrity and/or ion homeostasis (Table 1), and

* Corresponding author. Mailing address for Mohan K. Balasubramanian: Temasek Life Sciences Laboratory, 1 Research Link, The National University of Singapore, Singapore 117604. Phone: (65) 6872 7478. Fax: (65) 6872 7012. E-mail: mohan@tll.org.sg. Mailing address for Jianhua Liu: Genome Institute of Singapore, 60 Biopolis Street, no. 02-01, Singapore 138672. Phone: (65) 6478 8123. Fax: (65) 6478 9003. E-mail: liujh@gis.a-star.edu.sg.

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‡ A.B. and Y.J. contributed equally to this work.

§ Present address: Laboratoire de Virologie Moléculaire et Structural, CNRS, F-91198 Gif-sur-Yvette, France.

¶ Present address: Department of Oncological Sciences, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1130, New York, NY 10029.

TABLE 1. All 106 protein kinases in the *S. pombe* genome

Gene symbol	Systematic name	Viability	Process	Reference[a]
ark1	SPCC320.13c	Lethal	Mitosis and cytokinesis	46
bub1	SPCC1322.12c	Viable	Spindle checkpoint	7; this study[b]
byr1	SPAC1D4.13	Viable	Response to pheromone	40; this study
byr2	SPBC1D7.05	Viable	Response to pheromones	40; this study
cdc2	SPBC11B10.09	Lethal	Cell cycle regulation	5
cdc7	SPBC21.06c	Lethal	Mitosis and cytokinesis	15
cdk9	SPBC32H8.10	Lethal	Mitosis	45; this study
cdr1	SPAC644.06c	Viable	Cell cycle regulation	78; this study
cdr2	SPAC57A10.02	Viable	Cell cycle regulation	78; this study
cds1	SPCC18B5.11c	Viable	DNA replication checkpoint	38; this study
cek1	SPCC1450.11c	Viable	Unknown	55; this study
chk1	SPCC1259.13	Viable	DNA replication checkpoint	71; this study
cka1	SPAC23C11.11	Lethal	Polarized growth	62
cki1	SPBC1347.06c	Viable	Unknown	72; this study
cki2	SPBP35G2.05c	Viable	Unknown	72; this study
cki3	SPAC1805.05	Viable	Unknown	72; this study
cmk1	SPACUNK12.02c	Viable	Regulation of cell shape	48; this study
cmk2	SPAC23A1.06c	Viable	Response to oxidative stress	48; this study
crk1	SPBC19F8.07	Lethal	Regulation of transcription	10
csk1	SPAC1D4.06c	Viable	Cell cycle regulation	36; this study
dsk1	SPBC530.14c	Viable	Regulation of pre-mRNA splicing	64; this study
fin1	SPAC19E9.02	Viable	Mitosis and cellular morphogenesis	27; this study
gad8	SPCC24B10.07	Viable	Unknown	34; this study
gsk3	SPAC1687.15	Viable	Mitosis and cytokinesis	47; this study
gsk31	SPBC8D2.01	Viable	Unknown	77; this study
hhp1	SPBC3H7.15	Viable	DNA repair	13; this study
hhp2	SPAC23C4.12	Viable	DNA repair	19; this study
hri1	SPAC20G4.03c	Viable	Negative regulator of translation initiation	80; this study
hri2	SPAC222.07c	Viable	Negative regulator of translation initiation	80; this study
hsk1	SPBC776.12c	Lethal	Chromosome organization	33
kin1	SPBC4F6.06	Viable	Cell wall organization	29; this study
ksg1	SPCC576.15c	Lethal	Cell wall organization	41
lkh1	SPAC1D4.11c	Viable	Response to oxidative stress	25; this study
lsk1	SPAC2F3.15	Viable	Mitosis and cytokinesis	23a; this study
mde3	SPBC8D2.19	Viable	Sporulation	1; this study
mek1	SPAC14C4.03	Viable	Meiotic recombination	59; this study
mik1	SPBC660.14	Viable	DNA replication checkpoint	28; this study
mkh1	SPAC1F3.02c	Viable	Cell wall organization	57; this study
mph1	SPBC106.01	Viable	Mitotic spindle checkpoint	17; this study
nak1	SPBC17F3.02	Lethal	Polarized growth and maintenance	20; this study
oca2	SPCC1020.10	Viable	Unknown	65; this study
orb6	SPAC821.12	Lethal	Regulation of cell shape	68
pck1	SPAC17G8.14c	Viable	Cell wall organization	26; this study
pck2	SPBC12D12.04c	Viable	Cell wall organization	26; this study
pef1	SPCC16C4.11	Viable	Regulation of G_1/S transition	66; this study
pek1	SPBC543.07	Viable	Cell wall integrity	63; this study
pit1	SPAC3C7.06c	Viable	Sporulation	1; this study
pka1	SPBC106.10	Viable	Regulation of glycolysis	79; this study
plo1	SPAC23C11.16	Lethal	Mitosis and cytokinesis	43
pmk1	SPBC119.08	Viable	Cell wall integrity maintenance	67; this study
pom1	SPAC2F7.03c	Viable	Polarized growth and cytokinesis	2; this study
ppk1	SPAC110.01	Viable	Unknown	This study
ppk2	SPAC12B10.14c	Viable	Unknown	This study
ppk3	SPAC15A10.13	Viable	Unknown	This study
ppk4	SPAC167.01	Viable	Unknown	This study
ppk5	SPAC16C9.07	Viable	Unknown	This study
ppk6	SPAC1805.01c	Viable	Unknown	This study
ppk8	SPAC22G7.08	Viable	Unknown	This study
ppk9	SPAC23H4.02	Viable	Unknown	This study
ppk10	SPAC29A4.16	Viable	Unknown	This study
ppk11	SPAC2C4.14c	Viable	Unknown	This study
ppk13	SPAC3H1.13	Viable	Unknown	This study
ppk14	SPAC4G8.05	Viable	Unknown	This study
ppk15	SPAC823.03	Viable	Unknown	This study
ppk16	SPAC890.03	Viable	Unknown	This study
ppk18	SPAPB18E9.02c	Viable	Unknown	This study
ppk19	SPBC119.07	Viable	Unknown	This study

Continued on following page

TABLE 1—*Continued*

Gene symbol	Systematic name	Viability	Process	Reference[a]
ppk20	SPBC16E9.13	Viable	Unknown	This study
ppk21	SPBC1778.10c	Viable	Cytokinesis	A.B., J.L., and M.K.B., unpublished data
ppk22	SPBC1861.09	Viable	Unknown	This study
ppk23	SPBC18H10.15	Viable	Unknown	This study
ppk24	SPBC21.07c	Viable	Unknown	This study
ppk25	SPBC32C12.03c	Viable	Unknown	This study
ppk26	SPBC336.14c	Viable	Unknown	This study
ppk27	SPBC337.04	Viable	Unknown	This study
ppk28	SPBC36B7.09	Viable	Unknown	This study
ppk29	SPBC557.04	Viable	Unknown	This study
ppk30	SPBC6B1.02	Viable	Unknown	This study
ppk31	SPBC725.06c	Viable	Unknown	This study
ppk32	SPBP23A10.10	Viable	Unknown	This study
ppk33	SPCC162.10	Viable	Unknown	This study
ppk34	SPCC1919.01	Viable	Unknown	This study
ppk35	SPCC417.06c	Viable	Unknown	This study
ppk36	SPCC63.08c	Viable	Unknown	This study
ppk37	SPCC70.05c	Lethal	Unknown	This study
ppk38	SPCP1E11.02	Viable	Unknown	This study
prp4	SPCC777.14	Lethal	Regulation of pre-mRNA splicing	52
psk1	SPCC4G3.08	Viable	Unknown	37; this study
ran1	SPBC19C2.05	Lethal	Negative regulator of meiosis	6
sck1	SPAC1B9.02c	Viable	Cell proliferation	23; this study
sck2	SPAC22E12.14c	Viable	Unknown	15a; this study
shk1	SPBC1604.14c	Lethal	Regulation of cell shape	31
shk2	SPAC1F5.09c	Viable	Regulation of cell shape	76; this study
sid1	SPAC9G1.09	Lethal	Mitosis and cytokinesis	4
sid2	SPAC24B11.11c	Lethal	Mitosis and cytokinesis	4
spk1	SPAC31G5.09c	Viable	Meiosis	40; this study
spo4	SPBC21C3.18	Viable	Meiosis	39; this study
srb10	SPAC23H4.17c	Viable	Negative regulator of transcription	73; this study
srk1	SPCC1322.08	Viable	Negative regulator of meiosis	61; this study
ssp1	SPCC297.03	Viable	Cell wall organization	35; this study
ssp2	SPCC74.03c	Viable	Unknown	This study
sty1	SPAC24B11.06c	Viable	Response to stress	60; this study
wee1	SPCC18B5.03	Viable	Cell cycle regulation	42; this study
win1	SPAC1006.09	Viable	Response to stress	54; this study
wis1	SPBC409.07c	Viable	Response to stress	56; this study
wis4	SPAC9G1.02	Viable	Response to stress	58; this study

[a] Only one reference for each previously characterized protein kinase was selected due to space limitations. Additional references can be found in the PomDB database (www.incyte.com).
[b] A strain bearing a kinase deletion allele was constructed, and its phenotype was assessed in this study.

these mutant cells exhibit sensitivities to a variety of cell wall degrading agents (see Table 1 for references).

DNA microarray technology has aided analyses of gene expression in parallel on a genomic scale (12). It has been proposed that the genome-wide expression profile or signature of mutant cells could serve as a phenotypic characteristic for functional studies and/or for monitoring the signaling and circuitry of MAPK pathways (32, 50). Application of microarray technology to study signaling networks could permit dissection of pathways in great detail.

While a number of studies have focused on individual protein kinases that function in the regulation of numerous biological processes, including cellular responses to various stress factors, a systematic approach has not been applied to study the functions of all the protein kinase domain-containing proteins in response to different stresses in fission yeast. In this study, we report a systematic deletion analysis of genes encoding eukaryotic protein kinase catalytic-domain-containing proteins. The fission yeast genome (74) encodes a total of 106 eukaryotic protein kinase catalytic-domain-containing genes, about half of which have previously been characterized (Table 1). Three previously uncharacterized putative protein kinases were revealed, in this study, to be essential for vegetative growth. The addition of these 3 kinases to the 14 previously characterized kinases results in the classification of 17 protein kinases as being essential, out of a total of 106 kinases in the entire fission yeast genome. The remaining 89 dispensable genes have been individually deleted, and growth phenotypes of viable mutants assessed. This has revealed that half the total number of mutants exhibit hypersensitivity to at least one of the 17 stress factors tested. A comparison of the pattern of hypersensitivity to various stresses permitted the functional grouping of all putative protein kinases and suggested four major signaling pathways or networks that appear to be mediated by known and putative protein kinases in response to a variety of stress factors. Microarray analysis in a number of mutants has revealed a significant correlation of expression signatures in mutants with their growth phenotype, enabling a

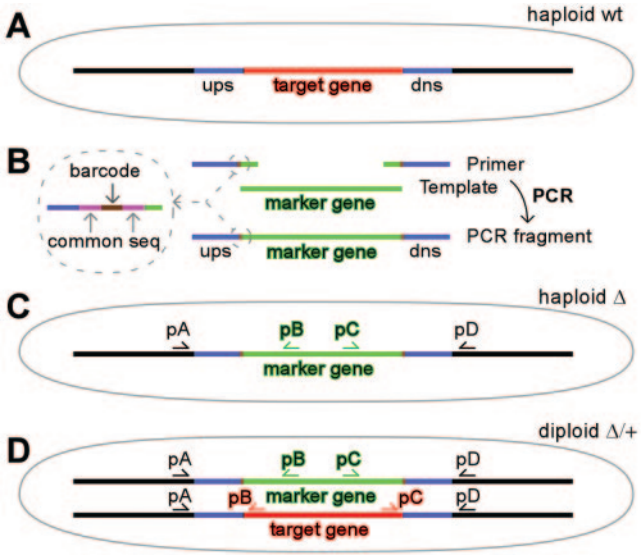

FIG. 1. Strategy for deletion allele construction. (A) Cell containing a wild-type target gene. (B) Pair of primers containing flanking sequences of a target gene was used to synthesize a DNA fragment by PCR, using a template carrying a selective marker gene. The barcode embedded into the long PCR primers has not been discussed in this study. (C) Haploid cell containing a deletion allele. (D) Diploid cell containing a heterozygous deletion allele. The target gene is indicated in red, and the marker gene is in green (in this study, the marker gene is *ura4*$^+$). wt, wild type; ups, upstream; dns, downstream; seq, sequence.

useful approach for a functional classification of protein kinases.

MATERIALS AND METHODS

Strains and media. Strains used in this study are listed in Table 1. The genotypes of parental strains are as follows: for MBY 192, *ura4-D18 leu1-32 h*$^-$; for MBY 1238, *ade6-210 ura4-D18 leu1-32 h*^{90}; for MBY 1239, *ade6-216 ura4-D18 leu1-32 h*^{90}; and for MBY1270, *ade6-210*/*ade6-216 ura4-D18*/*ura4-D18 leu1-32*/*leu1-32 h*^{90}/*h*^{90}. Standard media such as solid or liquid YES and EMM media (36a) were utilized with or without stress factors as indicated below. For sporulation, tests were performed using solid yeast extract-peptone-dextrose (YPD) medium.

Construction of deletion mutants. A PCR-mediated deletion approach (3) was used to construct targeted open reading frame (ORF) deletion mutants (Fig. 1). In brief, each synthesized primer (Integrated DNA Technologies, Coralville, IA) contained 80 nucleotides complementary to a particular ORF's flanking regions, followed by a barcode (20 nucleotides in length) and finally 18 nucleotides homologous to a uracil selection marker cassette. About 10 μg of PCR fragment was transformed into haploid or diploid cells using the lithium acetate method (24). Subsequently *ura4*$^+$ colonies were selected on EMM plates lacking uracil. Colonies were further examined using allele-specific primer pairs (see Table S1 in the supplemental material) to ensure deletion and replacement of the target gene by the *ura4*$^+$ gene.

Growth assay for hypersensitivity to stress factors. About 5 μl of log-phase cultures in a series of 10-fold dilutions was inoculated on solid YES media containing various stress factors using a multiblot replicator (V & P Scientific Inc., San Diego, CA). The growth of each mutant strain at various dilutions (factors of 0, 1, 2, 3, . . .) was monitored after 3 days of incubation at 28 to 30°C or as otherwise mentioned. Mutant phenotypes based on the ability of cells to grow at the highest dilution compared to that of wild-type cells was used to characterize cultures as not sensitive, sensitive, or hypersensitive. That is, mutants exhibiting growth comparable to that of wild-type cells at the same dilution were designated as "not sensitive" or "+"; mutants exhibiting growth similar to that of wild-type cells at approximately 1 dilution factor lower than that of wild-type cells were designated as sensitive or "-"; and mutants displaying growth comparable to that of wild-type cells at 2 (or greater) dilution factors lower than that of wild-type cells were designated as hypersensitive or "--" (or "---"). Except for temperature or minimal medium parameters, each stress factor was tested two to eight times at a range of concentrations (Table S2 in the supplemental material) that killed not more than 6% of the strains being tested or left more than 6% of the mutant strains unaffected. The average of sensitivities obtained from two to eight measurements was calculated and used as a phenotypic characteristic.

DNA damage responses. A number of mutants were assayed for sensitivity to UV-C and ionizing radiation using survival curves as previously described (69, 70). For testing sensitivities to radiomimetic drugs, exponential cultures were grown in YES medium, and 5 μl of cultures at various concentrations ranging from 10^6 to 10^3 cells/ml were spotted onto duplicate YES plates and incubated at 30°C for 3 days. Sensitivity was assayed relative to that on YES plates lacking the drug as described above.

Microarray analysis. Acid phenol was added to the frozen cell pellets, which were then incubated at 65°C for 15 min with occasional shaking using a thermomixer (Eppendorf, Hamburg, Germany). The aqueous phase was recovered by spinning at 5,000 rpm for 5 min at 4°C and reextracted by phenol-chloroform (1:1). RNA was precipitated by the addition of an equal volume of isopropanol and recovered by centrifugation at 10,000 rpm for 20 min at 4°C. Fluorescently labeled cDNA was synthesized using SuperScriptII (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with oligo$(dT)_{20}$ (Invitrogen) as primers in the presence of either Cy3- or Cy5-coupled dUTP (Invitrogen). The reaction was stopped by the addition of EDTA. The cDNA was subsequently hydrolyzed with NaOH and neutralized by HCl. Fluorescence-labeled cDNA was then washed and concentrated using microcon-YM30 spin columns (Millipore, Billerica, MA).

Spotted microarray slides were prehybridized using digoxigenin hybridization buffer (Roche, Basel, Switzerland) with a raised coverslip (Erie Scientific, Portsmouth, NH) in a slide hybridization chamber (GeneMachines) for 1 h at 42°C. Slides were washed first in distilled H_2O for 2 min and then in isopropanol for 2 min and spin dried. Microarray slides were hybridized using digoxigenin hybridization buffer containing Cy3- and Cy5-labeled cDNA overnight at 42°C. Hybridized slides were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 1 min, in 1× SSC for 4 min, in 0.2× SSC for 4 min, and in 0.05× SSC for 1 min and spin dried. Microarray slides were scanned using a GenePix scanner (Axon Instruments, Union City, CA) at 635-nm and 532-nm wavelengths at a resolution of 10 μm using GenePix Pro3 or Pro4 software (Axon Instruments). Data from each GenePix result file were normalized based on a median of ratios. The ratios of each spot were collected only if the intensity of the spots in either channel was ≥2-fold greater than the background.

Phylogenic analysis of protein kinases. Primary amino acid sequences of 106 known and putative protein kinases were obtained from databases (www.genedb.org and www.incyte.com). The catalytic-domain sequences of each protein kinase were extracted based on the Pfam definition for eukaryotic protein kinase catalytic domain (www.pfam.org). Alignment of catalytic-domain sequences and phylogenetic tree construction were carried out using Clustal W (18). The length of the dendrogram was unified for visualization.

Analyses of putative orthologs. Mutual best hit (MBH) analysis (http://www.ncbi.nlm.nih.gov/Homology/ComMapDoc.html) using BLAST (version 2.2.6) was utilized for identification of *Schizosaccharomyces pombe* protein kinase orthologs in *Saccharomyces cerevisiae* and human systems. A total of 106 eukaryotic protein kinase catalytic-domain sequences in *S. pombe* were compared to 119 sequences in *S. cerevisiae* and 491 sequences in humans as documented in the KinBase database (http://198.202.68.14/kinbase). Furthermore, a relaxed mutual best hit (repeated application of mutual best hit or RMBH) protocol was applied for identification of non-MBH orthologs in *S. cerevisiae* and human. In each iteration, a set of orthologs were identified using MBH analysis and removed from both genomes. In the next iteration, the updated genomes were submitted to MBH analysis for another set of orthologs and the procedure continued till either of the genomes was empty or no more mutual best hits were found. The minimum BLAST score threshold of 206 was set on the orthologs obtained from the second iteration of RMBH analysis. This threshold value was obtained as the score that covered 80% of all the orthologs obtained using MBH analysis only.

RESULTS

Evolutionary analysis of fission yeast protein kinases. A total of 106 genes encoding eukaryotic protein kinase catalytic-domain-containing proteins were found in the entire *S. pombe* genome based on the GeneDB (www.genedb.org) and the

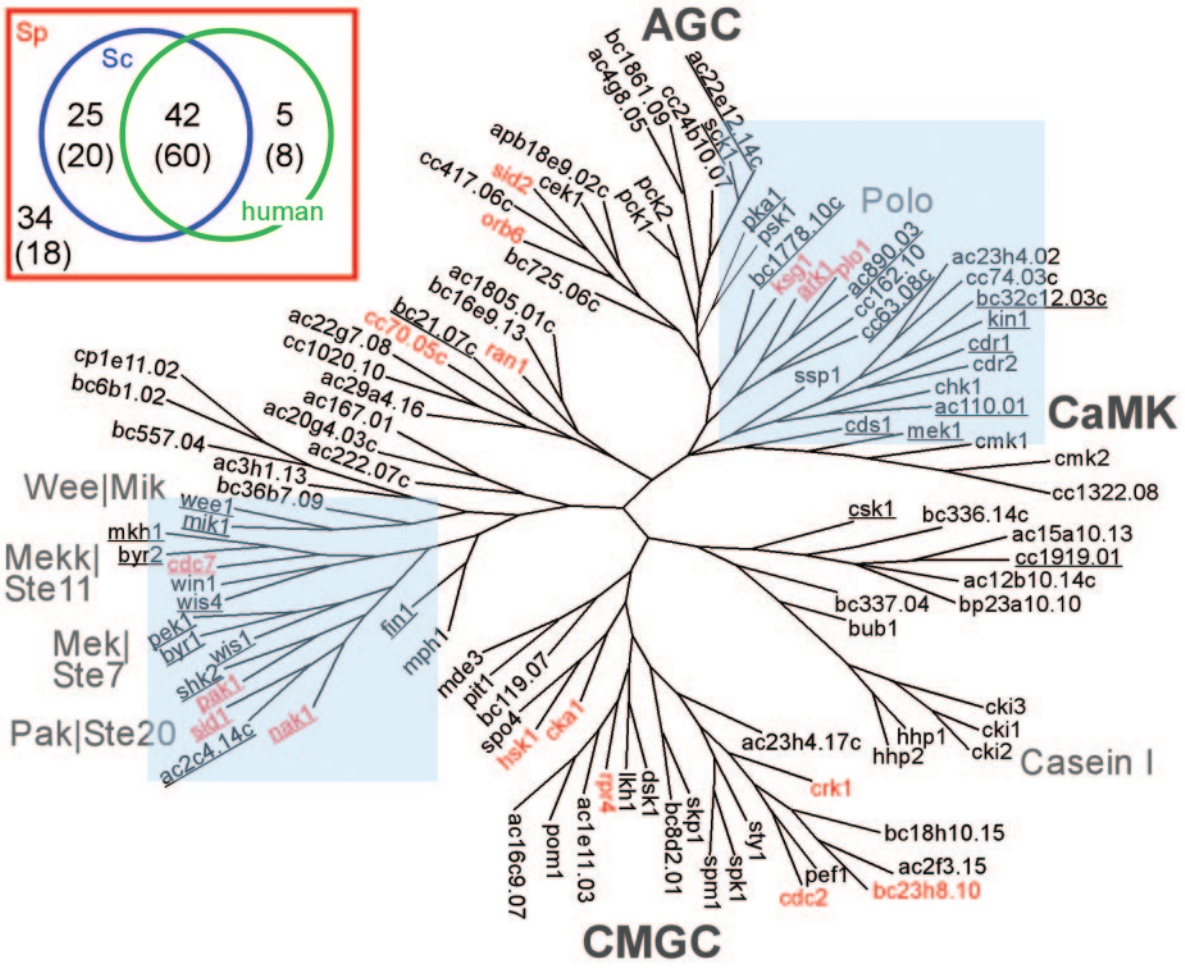

FIG. 2. Unrooted phylogenic tree of the 106 protein kinases in *S. pombe*. Seventeen essential protein kinases are marked in red, and 31 kinases containing tyrosine phosphorylation signatures are underlined. Two regions of the phylogenic tree shaded blue indicate that the region is enriched with tyrosine kinase signatures. AGC, CaMK, and CMGC indicate protein kinase groups, and Polo, Casein I, Wee/Mik, Mekk/Ste11, Mek/Ste7, and Pak/Ste20 indicate protein kinase families that do not belong to the AGC, CaMK, and CMGC groups. The inset shows a schematic representation of protein kinase orthologs in *S. pombe* (Sp), *S. cerevisiae* (Sc), and human. One hundred six eukaryotic protein kinase catalytic-domain-containing proteins were selected in *S. pombe*, 119 in *S. cerevisiae*, and 491 in human. Analysis of orthologs showed that of 106 *S. pombe* protein kinases, 67 (25 plus 42) have orthologs in *S. cerevisiae* and 47 (42 plus 5) in human. Among these, 42 appeared to have orthologs in both *S. cerevisiae* and human. Numbers in parentheses indicate the numbers of nearest homologs.

pombeDB (www.incyte.com/proteome) databases, of which approximately 45 were uncharacterized putative protein kinase (*ppk*) genes (Table 1). During the course of this project, some putative protein kinases were reported and have therefore been renamed accordingly. Phylogenic analysis of the protein kinases based on the catalytic-domain sequences (16, 22) revealed that no known or putative protein kinases in fission yeast belong to the PTK group, which consists of conventional protein tyrosine kinases (Fig. 2). Members of the PTK group are therefore likely to be specific to multicellular organisms, where they function in cell-cell communication (51). Although no PTK protein kinases could be detected in the *S. pombe* genome, 31 protein kinases possess a tyrosine kinase signature (www.ebi.ac.uk/interpro) suggesting a potential tyrosine kinase activity as shown in Fig. 2. Two previously well-characterized protein kinases, Rad3p and Tel1p, have been shown to possess a lipid-kinase domain instead of a protein-kinase domain, and therefore they are not discussed in this study.

Of the 106 known and putative protein kinases, 67 were found to have orthologs in a distant relative of *S. cerevisiae* and 47 in human, which increased to 80 in *S. cerevisiae* and 68 in human upon inclusion of the nearest homologs based on 106 protein kinase catalytic domain-containing proteins in *S. pombe*, 119 in *S. cerevisiae*, and 419 in human (Fig. 2, inset). According to MBH analysis, only 34 (or 18 based on RMBH analysis) out of 106 known and putative protein kinases were revealed to be unique to *S. pombe*. Forty-four percent (or 64%) of kinases in the entire genome appeared to have orthologs (or nearest homologs) in humans, indicating that studies on biological functions of fission yeast protein kinases would facilitate our understanding of protein kinase-mediated signaling pathways in human.

Putative protein kinases essential for viability. We investigated the functions of all putative protein kinases found in the *S. pombe* genome through a systematic deletion analysis. At the time we started this study, 14 protein kinases were known

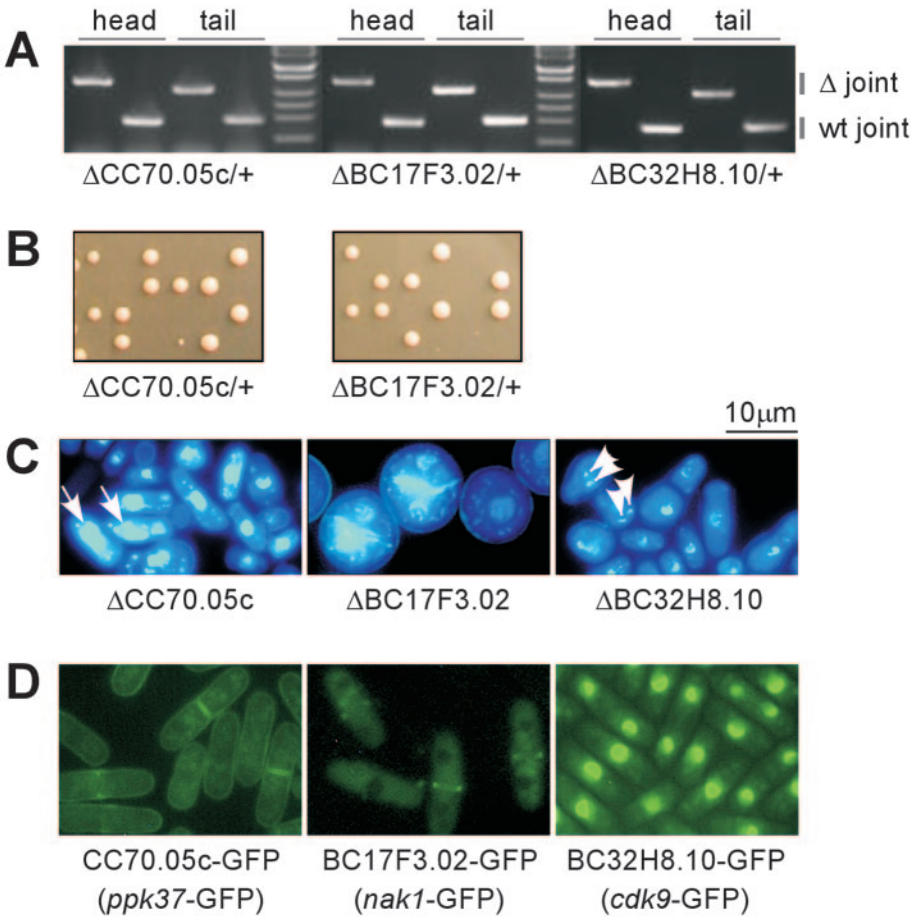

FIG. 3. Essential putative protein kinases. (A) Heterozygous deletion alleles in diploid mutant cells were verified by PCR using allele-specific primers as explained in the legend to Fig. 1. Wild-type (wt) alleles were confirmed by PCR using the target-specific primer pairs pA-pB for the 5′-end (head) joint and pC/pD for the 3′-end (tail) joint; deletion alleles were confirmed by PCR using primer pairs of target-specific pA–marker-specific pB-ura (head) and marker-specific pC-ura–target-specifc pD (tail). (B) Tetrad analysis confirmed that the putative protein kinases were essential for vegetative growth. (C) Free-spore assay of the mutants. DNA was stained using the fluorescent dye DAPI. Arrows and arrowheads indicate horse tail-like and condensed chromosomes, respectively. (D) Subcellular localization of GFP-tagged kinases in wild-type haploid cells. See the text for details.

to be essential for viability. We therefore selected the remaining 92 genes encoding known and putative protein kinases for the construction of deletion mutants (Table 1).

Individual deletion of three genes out of 92, namely, SPCC70.05c, SPBC17F3.02, and SPBC32H8.10, was not accomplished when haploid strains were transformed, suggesting that these gene products might be essential for cell viability. To further examine the essentiality of these putative protein kinases, we constructed deletion alleles in diploid cells. Verification of diploid mutants with a heterozygous deletion allele was performed via a PCR-based assay using two primer pairs, which were specific either for the wild-type allele or the deletion allele (Fig. 3A). The diploid mutant cells were subsequently grown on YPD plates for sporulation to segregate heterozygous alleles. Tetrad dissection of spores from asci generated from the heterozygous diploid mutants displayed a 2:2 segregation pattern, indicating that spores bearing a deletion allele were unable to form colonies (Fig. 3B). This was subsequently confirmed by replication of tetrad colonies onto a uracil drop-out plate. Spores carrying a deletion allele failed to form colonies, confirming that these three putative protein kinases were essential for viability.

Free-spore germination analysis of the three mutants revealed the following phenotypes. SPCC70.05c (*ppk*37) mutant cells showed an abnormal nuclear DNA morphology in that the DAPI (4′,6′-diamidino-2-phenylindole)-stained nuclear material was more diffuse and spread over a larger region of the cell (Fig. 3C), than that of a wild-type control. In some instances, these DNA structures appeared to resemble meiotic horse tail chromosomes. However, more work with conditional alleles will be required to establish the basis of this nuclear morphology phenotype. SPBC17F3.02 (allelic with *nak1*) mutants have a round cell morphology, indicating a defect in polarity establishment and/or maintenance; and SPBC32H8.10 mutants exhibit small-dot-like DNA staining, suggesting a compaction of chromosomal DNA in interphase cells (Fig. 3C).

We then further investigated the subcellular localization of these three essential putative protein kinases by tagging them with a green fluorescent protein (GFP) in wild-type cells. Hap-

loid cells bearing a putative protein kinase with GFP fused at its C terminus as a sole copy in the genome were viable, indicating that the tagged proteins were functional. Fluorescence microscopy analysis revealed distinct subcellular localization of these protein kinases: SPCC70.05c-GFP was predominantly distributed at cortical membranes, SPBC17F3.02-GFP was localized in the cytoplasm and at the division septum, and SPBC32H8.10-GFP showed a nuclear localization (Fig. 3D). During the process of this study, two *S. pombe* protein kinases, SPBC17F3.02/*nak1* and SPBC32H8.10/*cdk9*, were reported by Huang et al. (20) and Pei and Shuman (45). Both kinases have orthologs in *S. cerevisiae*: YHR102W/*KIC1* and YPR161C/*SGV1*.

Hypersensitivity of deletion mutant strains. Besides the three putative protein kinases that were found to be essential for viability, SPAC29A4.16 (*ppk10*) was initially unable to form colonies when deleted in haploid cells. Tetrad dissection analysis of a diploid knockout strain showed that all spores from tetrads, containing either a wild-type allele or a deletion allele, were viable and formed colonies on a YES plate. Examination of colonies replicated onto EMM plates lacking uracil revealed that *ura4*$^+$ colonies were very sensitive to minimal medium, which explained why the haploid deletion mutants failed to grow on the selective medium, suggesting a role for *ppk10* in some aspects of cellular metabolism.

Next we assessed the growth phenotype of all viable deletion mutants. At least two independent isolates were examined to ascertain that there was no random mutation in the background which could potentially alter the mutant phenotype. We also constructed deletion alleles of all the previously characterized dispensable protein kinases as a control for assessment of possible phenotypic alterations. The growth assay performed in this study (Table 2) indicated that the phenotypes of deletion mutants lacking a known protein kinase were identical to what was previously reported. Hence, we are confident that our putative protein kinase mutant strains constructed in haploid cells are unlikely to harbor any random mutations or suppressors.

The vast majority of nonessential kinase deletion mutants resembled wild-type cells in morphology, although a subset of previously characterized mutants (such as pom1, pck1, and pck2) exhibited abnormal morphology. In addition, several previously characterized mutants (wee1, cdr1, cdr2, wis1, spk1, and sty1) exhibited altered size at division. One kinase mutant (*pdk1*Δ; ppk21) was moderately defective in cytokinesis and will be published separately (A.B., J.L., and M.K.B., unpublished data).

The growth of all viable protein kinase deletion mutants was investigated under various stress conditions. Their temperature sensitivity was tested on YES plates incubated at 19°C (low temperature) and 36°C (high temperature). In order to determine the sensitivity of deletion mutant strains to various stress factors, we supplemented YES medium with common stress factors, such as salt (KCl), an osmolyte (sorbitol), an oxidant (hydrogen peroxide), agents that inhibit DNA replication (hydroxyurea [HU]) or damage DNA (ethyl methanesulfonate [EMS]), cytoskeleton poisons (methyl 2-benzimidazole carbamate [MBC], thiobendazole [TBZ], and latrunculin A [LatA]), chemicals that disrupt cell wall/membrane structures (calcofluor, sodium dodecyl sulfate), and agents that block the secretory pathway (brefeldin A), protein biosynthesis (cycloheximide), and chelate Ca^{2+} (EGTA).

For each stress factor, ranges of dosages were applied. For further analysis we chose only those concentrations at which no more than 6% of mutants displayed wild-type growth dynamics and at least 6% of mutants were able to grow. The ability of kinase deletion strains to grow at 36°C and on plates supplemented with TBZ or HU as an example is shown in Fig. 4, while Table 2 contains a summary of all data obtained from the sensitivity assays.

Functional grouping of putative protein kinases. To classify mutants based on similar growth defects under various stress conditions, we selected 41 mutants that exhibited hypersensitivity to at least one of the 17 different stress conditions tested and investigated the growth patterns of these strains under various stress conditions based on semiquantitative values of the mutant growth (Table 2). Two-dimensional cluster analysis using the Pearson correlation (14) with complete linkage groups based on a minimal similarity of 50% of the growth pattern under various stress conditions resulted in eight groups (Fig. 5).

Mutants in group A showed hypersensitivity to hydroxyurea, a drug that blocks DNA replication, and to the mutagens methyl methanesulfonate and 4-nitroquinoline-*N*-oxide (4NQ) (Table 3). This is consistent with previous reports indicating that *cds1*$^-$, *chk1*$^-$, *hhp2*$^-$, and *mik1*$^-$ mutants are defective in DNA repair and/or DNA damage/replication checkpoint control (13, 28, 38, 71).

Most mutants in groups B and C exhibited multiple sensitivities to a number of stress factors, such as the microtubule poison TBZ, the actin-depolymerizing agent LatA, the replication inhibitor HU, the detergent SDS, and the antibiotic bleomycin. These protein kinases and putative protein kinases are likely to be involved in multiple biological processes, including cell cycle regulation, cell wall biogenesis, and polarized growth (see Table 1 for references).

Group D contained mutants such as the *wis4*$^-$, *win1*$^-$, and *sty1*$^-$ mutants, which exhibited hypersensitivity to a number of common stress factors, such as high temperature, high salt concentration, and oxidative stress, consistent with previous reports (54, 58, 60). Besides the *wis4*$^-$, *win1*$^-$ and *sty1*$^-$ mutants, two other kinases, the *kin1*$^-$ mutant and the previously uncharacterized *ppk19*$^-$ (BC119.07) mutant, could be clustered into the same group. Interestingly both the *kin1*$^-$ and *ppk19*$^-$ mutants in addition to the above-mentioned stress factors displayed hypersensitivity to a number of other conditions, i.e., EGTA and cycloheximide.

Mutants in group E showed diverse phenotypes and likely function in cell cycle regulation and cell growth, similar to those in groups B and C, and in response to common stresses, such as temperature and the presence of salt, an oxidant, and an osmolyte.

Mutants in group F exhibited hypersensitivity limited to microtubule poisons such as MBC and TBZ, implicating their involvement in noncytoplasmic microtubule-associated processes. This group includes molecules such as Fin1 and Bub1, which are known to regulate mitotic spindle assembly or spindle checkpoint control (7, 27). Many mutants in groups B, C, and E showed hypersensitivity to both microtubule poisons and to the actin inhibitor LatA. It is conceivable that mutations

TABLE 2. Summary of growth phenotypes of the deletion mutants lacking one of the nonessential known and putative protein kinases indicated

Gene symbol	Systematic name[b]	Growth with indicated stress[a]																
		19°C	36°C	MM	TBZ	MBC	LatA	BFA	cHex	H_2O_2	HU	EMS	Bleo	SDS	EGTA	KCl	Sorb	Calc
bub1	SPCC1322.12c	ND	+	ND	---	---	+	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND
byr1	SPAC1D4.13	+	+	-	-	-	+	+	-	+	+	+	-	+	+	+	+	+
byr2	SPBC1D7.05	+	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-
cdr1	SPAC644.06c	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+
cdr2	SPAC57A10.02	+	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	+
cds1	SPCC18B5.11c	+	+	-	-	+	-	+	-	-	---	+	+	-	+	-	+	+
cek1	SPCC1450.11c	-	+	-	-	-	-	+	+	+	+	+	+	--	+	-	+	-
chk1	SPCC1259.13	+	+	+	-	-	+	-	+	+	---	-	-	+	+	+	+	+
cki1	SPBC1347.06c	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
cki2	SPBP35G2.05c	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
cki3	SPAC1805.05	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
cmk1	SPACUNK12.02c	+	-	-	-	+	+	+	-	+	-	+	-	+	+	+	+	+
cmk2	SPAC23A1.06c	+	+	-	+	+	+	+	-	+	-	+	-	-	+	+	+	+
csk1	SPAC1D4.06c	---	+	---	--	--	-	--	-	-	-	+	--	--	+	-	-	-
dsk1	SPBC530.14c	+	+	-	-	-	+	+	-	+	-	-	-	+	-	+	+	+
fin1	SPAC19E9.02	+	+	+	---	---	+	+	+	+	-	+	+	+	+	+	+	+
gad8	SPCC24B10.07	-	+	+	---	-	-	+	+	-	+	+	--	--	+	--	+	+
gsk3	SPAC1687.15	-	+	+	-	-	-	+	+	+	+	+	+	-	+	-	+	+
gsk31	SPBC8D2.01	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+
hhp1	SPBC3H7.15	--	-	-	-	-	+	-	+	-	---	---	--	--	+	-	-	+
hhp2	SPAC23C4.12	+	+	+	-	+	+	+	+	+	--	-	+	+	+	-	+	+
hri1	SPAC20G4.03c	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	+
hri2	SPAC222.07c	+	+	+	-	+	+	+	+	-	+	-	-	-	+	-	+	-
kin1	SPBC4F6.06	+	-	-	---	--	+	-	---	+	-	-	---	--	---	--	+	+
lkh1	SPAC1D4.11c	-	-	-	-	-	-	+	-	-	-	+	-	--	+	-	-	-
lsk1	SPAC2F3.15	-	+	+	--	-	+	+	+	-	+	-	-	+	+	+	+	+
mde3	SPBC8D2.19	+	+	+	-	+	-	+	+	+	-	+	+	-	+	-	-	-
mek1	SPAC14C4.03	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
mik1	SPBC660.14	-	+	+	-	-	+	+	+	+	---	+	+	+	+	+	+	+
mkh1	SPAC1F3.02c	+	-	-	--	-	+	+	+	+	-	-	+	+	-	-	+	+
mph1	SPBC106.01	+	+	--	---	---	+	-	-	-	-	-	-	-	-	-	+	+
oca2	SPCC1020.10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pck1	SPAC17G8.14c	--	-	+	-	+	-	+	+	+	+	+	+	-	+	-	+	+
pck2	SPBC12D12.04c	---	--	+	-	-	+	+	+	-	-	+	+	-	+	-	-	+
pef1	SPCC16C4.11	-	-	---	-	+	-	-	+	--	-	-	--	-	-	-	+	-
pek1	SPBC543.07	+	+	-	--	-	-	+	+	+	+	+	+	+	+	+	+	+
pit1	SPAC3C7.06c	+	+	-	-	-	+	+	+	+	+	-	+	+	+	-	+	+
pka1	SPBC106.10	+	+	+	--	--	---	+	-	-	--	+	--	-	+	---	-	+
pmk1	SPBC119.08	-	-	-	--	-	--	-	+	-	-	-	-	-	-	-	-	+
pom1	SPAC2F7.03c	--	+	+	-	-	--	-	-	+	-	-	-	--	-	-	-	-
ppk1	SPAC110.01	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	+
ppk2	SPAC12B10.14c	ND	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+
ppk3	SPAC15A10.13	-	+	+	--	-	-	-	-	-	-	-	-	-	+	-	+	-
ppk4	SPAC167.01	+	-	--	-	-	-	-	+	+	-	-	-	-	+	-	-	-
ppk5	SPAC16C9.07	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+
ppk6	SPAC1805.01c	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
ppk7	SPAC22E12.14c	+	+	+	-	-	+	-	-	+	+	-	+	+	+	-	+	+
ppk8	SPAC22G7.08	+	+	+	-	+	+	+	-	+	-	-	-	+	-	+	+	+
ppk9	SPAC23H4.02	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+
ppk10	SPAC29A4.16	-	+	---	---	-	--	-	--	-	---	-	---	--	+	+	-	-
ppk11	SPAC2C4.14c	+	-	+	-	-	+	+	+	-	+	-	-	+	+	+	+	+
ppk13	SPAC3H1.13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ppk14	SPAC4G8.05	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
ppk15	SPAC823.03	-	+	+	-	+	-	-	+	-	-	+	--	--	+	+	+	-
ppk16	SPAC890.03	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+
ppk18	SPAPB18E9.02c	--	+	--	--	+	--	+	+	+	+	+	-	-	+	-	+	-
ppk19	SPBC119.07	--	---	-	---	-	+	+	--	-	+	+	---	+	---	--	+	+
ppk20	SPBC16E9.13	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
ppk21	SPBC1778.10c	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+	+	+
ppk22	SPBC1861.09	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	-	-
ppk23	SPBC18H10.15	--	+	+	-	+	-	+	+	-	-	+	-	-	+	-	-	-
ppk24	SPBC21.07c	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
ppk25	SPBC32C12.03c	-	+	+	-	-	-	-	+	+	-	-	+	+	+	-	+	+
ppk26	SPBC336.14c	+	-	--	--	-	+	+	+	+	+	-	+	-	-	+	-	+
ppk27	SPBC337.04	---	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+

Continued on following page

TABLE 2—*Continued*

Gene symbol	Systematic name	Growth with indicated stress[a]																
		19°C	36°C	MM	TBZ	MBC	LatA	BFA	cHex	H_2O_2	HU	EMS	Bleo	SDS	EGTA	KCl	Sorb	Calc
ppk28	SPBC36B7.09	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+
ppk29	SPBC557.04	-	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	-
ppk30	SPBC6B1.02	-	+	ND	-	-	-	+	+	-	+	+	-	-	-	+	-	+
ppk31	SPBC725.06c	+	+	+	-	-	+	+	+	+	+	--	+	+	-	+	+	+
ppk32	SPBP23A10.10	-	+	+	--	-	+	+	+	+	+	-	+	-	+	-	-	+
ppk33	SPCC162.10	+	+	-	-	-	-	+	-	+	-	-	+	+	+	+	+	-
ppk34	SPCC1919.01	-	+	-	-	+	+	-	+	+	+	+	--	-	+	+	+	-
ppk35	SPCC417.06c	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
ppk36	SPCC63.08c	+	+	+	-	+	-	+	+	+	-	-	+	+	+	+	+	-
ppk38	SPCP1E11.02	+	-	+	-	+	+	-	+	+	+	-	-	-	-	+	+	+
psk1	SPCC4G3.08	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+
sck1	SPAC1B9.02c	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
shk2	SPAC1F5.09c	+	+	--	-	+	-	+	+	+	-	+	-	+	+	+	+	-
spk1	SPAC31G5.09c	-	+	-	-	-	+	+	-	+	-	-	-	+	+	-	+	+
spo4	SPBC21C3.18	+	+	-	-	-	--	-	+	+	-	+	-	-	+	-	-	--
srb10	SPAC23H4.17c	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+
srk1	SPCC1322.08	+	+	-	-	-	-	-	+	+	-	+	-	+	-	-	+	-
ssp1	SPCC297.03	+	-	-	-	-	+	+	+	+	+	-	+	+	+	-	-	+
ssp2	SPCC74.03c	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
sty1	SPAC24B11.06c	+	---	--	--	-	+	-	-	---	-	-	--	-	-	---	--	+
wee1	SPCC18B5.03	---	--	-	---	--	--	-	--	-	--	+	---	--	+	-	-	-
win1	SPAC1006.09	+	---	+	--	-	+	+	+	-	+	-	+	-	+	--	-	-
wis1	SPBC409.07c	---	---	-	-	-	--	-	-	---	+	+	--	---	+	---	--	-
wis4	SPAC9G1.02	+	−−	+	-	+	-	-	-	--	-	-	-	-	-	--	+	-

[a] Abbreviations for stress factors: MM, minimum medium; TBZ, thiabendazole; MBC, methyl 2-benzimidazole carbamate; LatA, latrunculin A; BFA, brefeldin A; cHex, cyclohexmide; HU, hydroxyurea; EMS, ethyl methanesulfonate; Bleo, bleomycin; SDS, sodium dodecyl sulfate; Sorb, sorbitol; Calc, calcofluor. Description of phenotypes: ND, no data; +, wild-type growth; -, growth at 1 dilution factor less than that of wild-type cells; --, 2 dilution factors less; ---, 3 (or >3) dilution factors less than that of the wild-type cells.

affecting the function of protein kinases involved in cytoplasmic microtubule-mediated cellular growth would result in hypersensitivity to both microtubule poisons and polarized growth inhibitors.

Group G kinases appear to be involved in the assembly of the mitotic spindle, and in this respect they are similar to the members of group F described above. However, they have an additional role in maintaining cell wall integrity.

The last group, group H, is comprised of three members, individual deletions in which resulted in very poor growth at 19°C. Pck1 and Pck2 are known to be involved in cellular morphogenesis. The comparison of growth phenotypes thus allowed us to assign functions to putative protein kinases based on established information of previously characterized protein kinases classified in the same group (see references in Table 1).

We applied each of the stresses at an intermediate dosage range that resulted in comparable effects on mutant cell growth; that is, the dose was neither too strong to inhibit the growth of a majority of mutants (e.g., >6%) nor too weak to display any effect on many mutants (>6%). Given the fact that all available protein kinase mutants were tested under identical conditions, the different effects of stress factors could thus be compared. Following two-dimensional cluster analysis, 17 stress factors were separated into four categories (Fig. 5). Category 1 had only two factors, EGTA (Ca^{2+} chelate) and cycloheximide (protein biosynthesis inhibitor), and only two mutants, the *ppk19*$^-$ and *kin1*$^-$ mutants, exhibited hypersensitivity to these factors. Category 2 consisted of the microtubule poisons TBZ and MBC, and agents affecting DNA replication and integrity viz., HU and EMS, respectively. In this study, we found that EMS was less potent than methyl methanesulfonate (MMS); therefore, we tested a number of mutants using the stress factors MMS and 4NQ (Table 3). Those deletion mutants that showed hypersensitivity to HU exhibited hypersensitivity to both MMS and 4NQ. It is not clear why microtubule poisons and DNA damage (or replication block) agents grouped together. It may reflect a group of stress factors that could potentially destabilize genome integrity since both chromosomal DNA and mitotic spindles are nuclear and required for this process. Category 3 contains a number of factors that affect general cellular growth: calcofluor disrupts cell wall structures, latrunculin A prevents polymerization of the actin cytoskeleton, and brefeldin A interferes with secretory pathways. Category 4 contains those stress factors that are commonly present in the environment, such as high or low temperatures, high salt, a high osmolyte concentration, and the oxidant H_2O_2. Each category may reflect a major signaling pathway that is involved in the response to the stress factors within the category. Consistent with this, it is likely that Wis4-Wis1-Sty1 is the major signaling pathway in response to a number of common stress factors, such as temperature and the presence of salt, an osmolyte, and an oxidant (9).

Conjugation, meiosis, and sporulation. Fission yeast cells undergo meiosis upon nutritional starvation, leading to the formation of ascospores (75). A number of regulators, including protein kinases that are required for conjugation (e.g., Byr2, Byr1, and Spk1), meiosis (e.g., Mde3 and Mek1), and sporulation (e.g., Spo4 and Pit1) have been previously charac-

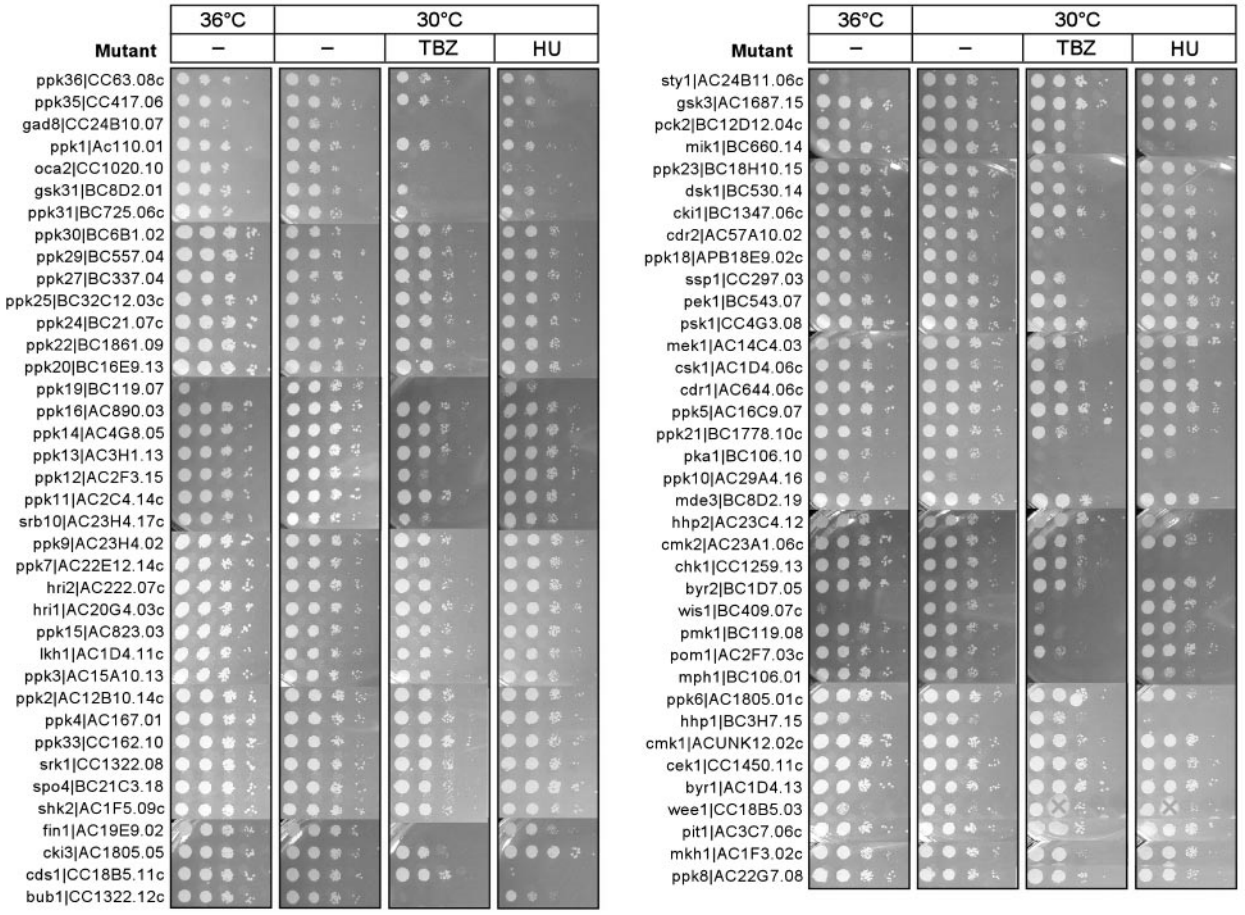

FIG. 4. Phenotypic assessment of nonessential protein kinase mutants. Approximately 5 μl of 10-fold serial dilutions of the 88 viable haploid h^- mutant cells (*sck1*$^-$ mutant was not available) were spotted and grown under 17 different stress conditions with various dosages. Examples of cell growth under stress conditions such as high temperature (36°C), a microtubule poison (TBZ), and an agent that blocks DNA replication (HU) are shown. A mutant was described as hypersensitive to a stress condition when its growth was similar to that of wild-type cells at 2 (or >2) dilution factors lower. Mutant growth phenotypes under various conditions are individually summarized in Table 2.

terized (see references in Table 1 and www.genedb.org/ and www.incyte.com/proteome/). Mutations in some of them (e.g., the *byr2*, *byr1*, *med3*, *mek1*, and *pit1* genes) were found to have no obvious vegetative growth phenotype under various stress conditions tested (Fig. 6) (see references in Table 1) but failed to conjugate or undergo meiosis or sporulation (Fig. 6). In order to identify protein kinases which are involved in any of these three processes, we constructed 84 individual kinase deletion alleles in a haploid homothallic (h^{90}) strain that could undergo mating and sporulation upon nitrogen starvation (75). Aliquots from a set of serially diluted cultures were spotted onto YPD plates for induction of sporulation. Wild-type cells formed spores after 3 to 4 days at 24°C as indicated by a dark-brown staining of spores upon a brief exposure to iodine vapor (data not shown) due to amyloid-rich spore walls (8). Of 84 protein kinase and putative protein kinase mutants tested, 15 showed major defects in spore formation based on iodine vapor staining and 10 showed partial defects (Fig. 6). Thus, about 30% of protein kinases found in the entire fission yeast genome were required for conjugation, meiosis, or sporulation.

Mapping signaling pathways using genome-wide expression signatures of protein kinase mutants. In this study, we found that 15 known and putative protein kinase mutants were hypersensitive to at least one of the stress factors tested and were also defective in conjugation, meiosis, or sporulation, while 12 mutants displayed only meiotic defects (Fig. 6). We therefore sought to ascertain whether protein kinase-mediated signaling pathways that play multiple roles in processes such as vegetative growth, conjugation, meiosis, and sporulation exist. It has been demonstrated that expression signatures are uniquely characteristic of various mutants (21). Using an oligonucleotide-based DNA microarray covering all predicted genes, we obtained genome-wide expression profiles or expression signatures from a number of protein kinase mutants (see Table S3 in the supplemental material) whose phenotypes upon deletion were well defined except for one putative kinase (*ppk19*). Pearson correlation analysis with complete linkage (14) demonstrated that single expression signatures of various protein kinase mutants could be clearly correlated with their growth phenotype during both vegetative growth and sporulation (Fig. 7).

As shown in Fig. 7, the expression signatures of mutant *sty1*$^-$, *wis4*$^-$, and *win1*$^-$ strains (mutants that are defective in the oxidative-stress response) clustered together, consistent

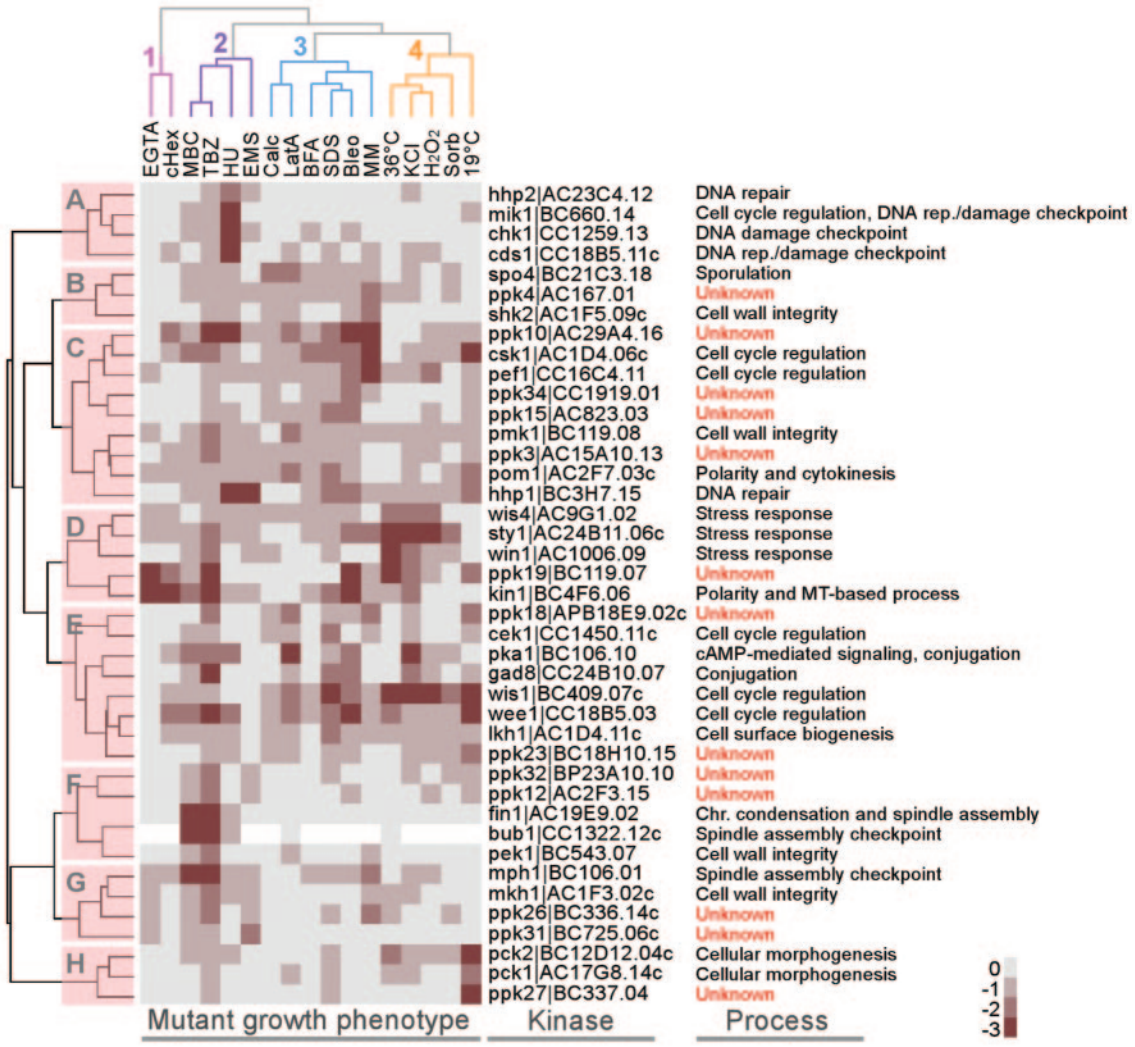

FIG. 5. Phenotypic clustering of hypersensitive mutants. Pearson correlation clustering of the growth pattern of the protein kinase mutants was performed to analyze 41 known and putative protein kinase mutants that exhibited hypersensitivity to at least one of the 17 stress conditions tested (see the text). Upon comparison of growth phenotypes under various conditions, 41 mutants were divided into eight groups with a minimal similarity of 50%. The 17 conditions used in the phenotypic assessment were separated into four clusters. rep., repair; MT, microtubule; Calc, calcofluor; BFA, brefeldin A; SDS, sodium dodecyl sulfate; Bleo, bleomycin; MM, minimum medium; Sorb, sorbitol.

with results obtained from phenotypic assessment (Fig. 5). Intriguingly, the expression signatures of the protein kinase *byr1*$^-$ and *spk1*$^-$ mutants (defective in conjugation) clustered together but could be distinguished from cell wall integrity *mkh1*$^-$ and *pek1*$^-$ mutants and oxidation stress-sensitive *sty1*$^-$, *wis4*$^-$, and *win1*$^-$ mutants. A simple expression signature could therefore identify the mutant phenotype of a strain. Furthermore, the cluster of *byr1*$^-$ and *spk1*$^-$ signatures was closer to that of *mkh1*$^-$ and *pek1*$^-$ signatures (Fig. 7) than to the location of the *sty1*$^-$, *wis1*$^-$, and *win1*$^-$ cluster. This was consistent with a common function for the *byr1-spk1* and the *mkh1-pek1* clusters in cellular morphogenesis. In addition, mutant *kin1* was known to be involved in microtubule-mediated polarized growth (29). In a phenotypic assessment, the *kin1*$^-$ mutant was grouped with the *sty1*$^-$, *wis4*$^-$, and *win1*$^-$ mutants (Fig. 5). However, based on the expression signature, the *kin1*$^-$ mutant was grouped with the SPBC119.07$^-$ mutant and closely

TABLE 3. Hypersensitivities of known and putative protein kinase mutants to DNA damage agents

Gene symbol	Systematic name	Growth[a] on:		Cell cycle arrest phenotype[b] on:	
		MMS	4NQ	MMS	4NQ
bub1	SPCC1322.12c	--	-	EI	EV
cds1	SPCC18B5.11c	---	---	EI	EI
chk1	SPCC1259.13	---	---	EI	EI
gad8	SPCC24B10.07	---	---	EI	EI
hhp1	SPBC3H7.15	---	---	EI	EI
kin1	SPBC4F6.06	--	-	EI	EV
mik1	SPBC660.14	---	---	EI	EI
oca2	SPCC1020.10	---	-	EI	EV
ppk19	SPBC119.07	---	--	EI	EV
ppk26	SPBC336.14c	--	+	EI	EV
wis1	SPBC409.07c	---	---	EI	EI
wee1	SPCC18B5.03	---	---	EI	EI

[a] + and - stand for growth as defined in Table 2, footnote *a*.
[b] Cell cycle arrest phenotypes were assessed after several generations on the stress indicated. EI and EV stand for elongated inviable and viable cells, respectively.

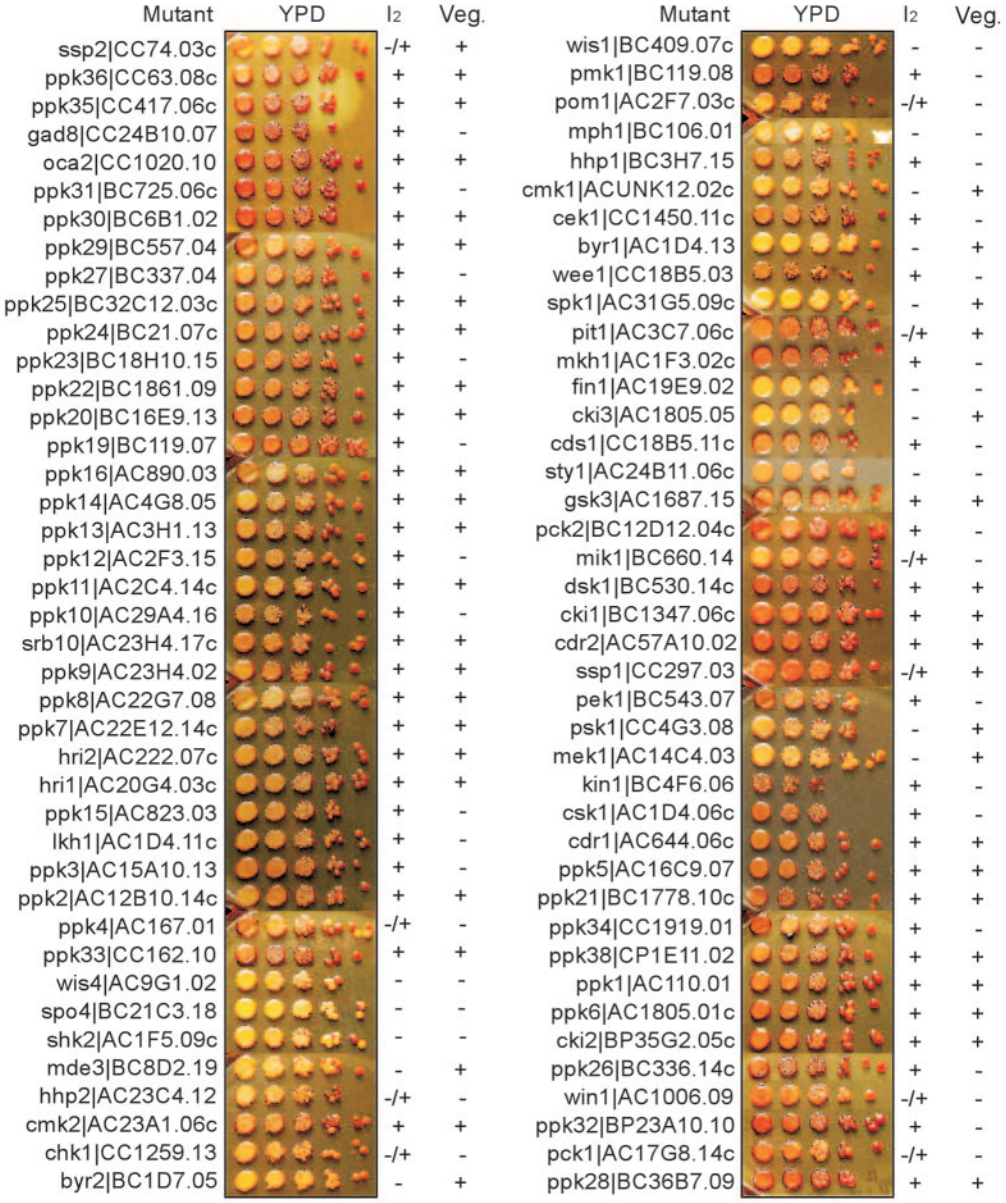

FIG. 6. Sporulation assay. Iodine staining of mutants under conditions promoting sporulation was performed. About 5 μl of 10-fold serial dilutions of 84 haploid h^{90} mutant cells (5 h^{90} mutants were not available) were tested for conjugation, meiosis, or sporulation. Mutants defective in conjugation, meiosis, or sporulation display negative staining with iodine vapor (I_2) and are indicated with "-." Partially negative stainings are marked with "-/+." Mutant cells which displayed the vegetative growth phenotype (Veg.) under at least one of the 17 conditions tested are indicated with "-" (see Fig. 5). A white crossed spot was a bacterial contaminant.

related to the *pek1* and *mkh1* mutants (Fig. 7). SPBC119.07 is likely to have a function similar to that of *kin1* since both phenotypic assay and expression profiling indicated that they are closely related. The expression signature of a mutant is based on transcriptional changes at a whole-genome level (21), whereas a phenotypic assay includes a limited set of selected conditions. The expression signature could therefore be extrapolated to predict the mutant phenotype (i.e., the cellular response to different stress factors) and finally could be utilized for the functional classification of all known and putative protein kinases.

DISCUSSION

Determining the effect of gene deletions is a fundamental approach to understanding gene function. We have carried out a systematic deletion analysis for all the putative protein kinases and known dispensable protein kinases in the fission yeast *S. pombe*. We identified three genes whose function is essential for cell viability (Fig. 3 and Table 1). Thus, the total number of fission yeast protein kinases indispensable for vegetative growth is 17 (16%). A phenotypic assessment of viable deletion mutants revealed that the function of 46% of these

Kinase	Mutant growth phenotype	Process
Byr1	No mitotic growth defect	Pheromone response
Mkh1	Hypersensitive to TBZ	Cell wall integrity
Pek1	Hypersensitive to TBZ	Cell wall integrity
Spk1	No mitotic growth defect	Pheromone response
Win1	Hypersensitive to common stress	Oxidative stress response
Wis4	Hypersensitive to common stress	Oxidative stress response
Sty1	Hypersensitive to common stress	Oxidative stress response
Kin1	Hypersensitive to TBZ	MT-based polarized growth
BC119.07	Hypersensitive to TBZ	Unknown

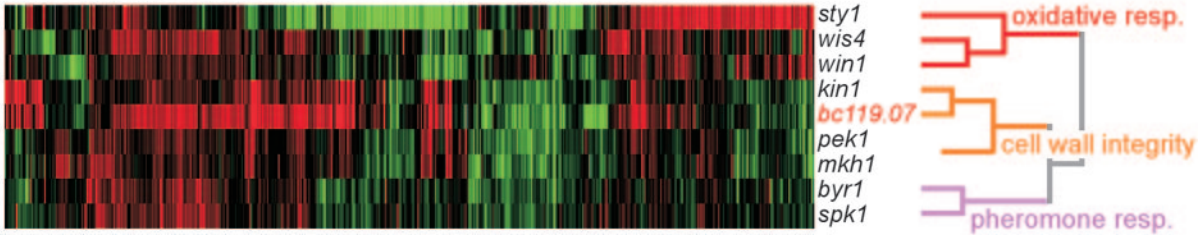

FIG. 7. Expression signatures identify mutant phenotypes. Pearson correlation clustering of the expression signatures of the protein kinase mutants was performed. An expression profile of each mutant under optimal growth conditions was attained using oligonucleotide-based whole-genome DNA microarrays with a wild-type strain (*ura4*$^+$) as a common reference. Eight hundred forty-five genes whose expression levels changed 1.6-fold or greater in at least one of the nine mutant profiles were selected for cluster analysis. Eight protein kinases are known to be involved in oxidative-stress responses (in red), cell wall integrity or polarized growth (in orange), and pheromone responses (in purple). A putative protein kinase, SPBC119.07, which showed growth defects in drug sensitivity assays similar to those of the *kin1* mutant (Fig. 5) displayed an expression signature similar to that of the *kin1* mutant. MT, microtubule.

kinases is required for growth under the stress conditions tested in this study.

Various stress factors could disrupt different cellular metabolic pathways and/or cellular components. Defective growth observed under a particular stress condition implies that the deletion kinase is potentially involved in mediating signals during the cellular response given to that stress factor. In addition, dosage ranges of stress factors exhibit different degrees of disruption to cellular growth. By assaying a large number of deletion mutants with a range of dosages, we have shown that the stress factors may be divided into four groups. This raises the possibility of the existence of four major signaling pathways or networks organized in fission yeast in response to the stress factors tested. This is consistent with the proposition by Chen et al. (9) that *wis4-wis1-sty1* is the major signaling pathway mediating signals in response to a number of stress factors in category 4.

Of 89 dispensable protein kinases, 15 are found to function both in vegetative growth under stress conditions and in sporulation, and 12 appear to function specifically in sporulation. Thirty-six out of 89 dispensable kinase mutants displayed no obvious phenotype in both the vegetative-growth assay and the sporulation assay, suggesting redundant functions for these protein kinases. Alternatively, they may be required for growth under conditions not examined in this study.

We have shown that single genome-wide expression signatures of individual mutants can be utilized to classify the protein kinases involved in oxidative stress and pheromone responses. Furthermore, it can effectively differentiate kinases that are involved in morphogenesis from those that do not play roles in this process. It is likely that the expression signature will reveal information about those kinases whose deletion mutants do not exhibit any detectable phenotype. The determination of expression signatures is potentially a very important approach for functional analysis of various protein kinase-mediated signaling pathways.

Eukaryotic protein kinase catalytic-domain-containing proteins are conserved from yeast to human. We have extracted 106 known and putative protein kinases from the entire *S. pombe* genome. Comparative analyses using mutual best-hit analysis and nearest-homolog analysis demonstrated that 44% and 64% of *S. pombe* protein kinases could be paired with their orthologues or nearest homologues in human, respectively (Fig. 2). Therefore, the study of protein kinase functions in fission yeast could promote our understanding of the biological functions of protein kinase-mediated signaling pathways in mammalian systems. We hope that our mutants and the results presented in this study will be useful to our colleagues in further elucidating protein kinase-mediated signaling pathways or networks in yeast as well as in higher eukaryotic systems. Our microarray data sets for expression signature of protein kinase mutants tested are available at http://giscompute.gis.a-star.edu.sg/~gisljh/kinome.

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Please address strain requests to M.K.B. and queries relating to microarray to J.L.

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