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Global Analysis of Serine-Threonine Protein Kinase Genes in *Neurospora crassa*▽†

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Serine/threonine (S/T) protein kinases are crucial components of diverse signaling pathways in eukaryotes, including the model filamentous fungus *Neurospora crassa*. In order to assess the importance of S/T kinases to *Neurospora* biology, we embarked on a global analysis of 86 S/T kinase genes in *Neurospora*. We were able to isolate viable mutants for 77 of the 86 kinase genes. Of these, 57% exhibited at least one growth or developmental phenotype, with a relatively large fraction (40%) possessing a defect in more than one trait. S/T kinase knockouts were subjected to chemical screening using a panel of eight chemical treatments, with 25 mutants exhibiting sensitivity or resistance to at least one chemical. This brought the total percentage of S/T mutants with phenotypes in our study to 71%. Mutants lacking *apg-1*, an S/T kinase required for autophagy in other organisms, possessed the greatest number of phenotypes, with defects in asexual and sexual growth and development and in altered sensitivity to five chemical treatments. We showed that NCU02245/*stk-19* is required for chemotropic interactions between female and male cells during mating. Finally, we demonstrated allelism between the S/T kinase gene NCU00406 and *velvet* (*vel*), encoding a p21-activated protein kinase (PAK) gene important for asexual and sexual growth and development in *Neurospora*.

Protein phosphorylation is a central component of numerous mechanisms that regulate critical cellular functions. In eukaryotic cells, signal transduction, metabolism, movement, the circadian rhythm, and many other processes are controlled through protein phosphorylation and dephosphorylation by protein kinases and phosphatases, respectively. Together with tyrosine kinases, serine-threonine (S/T) protein kinases comprise a large class in the eukaryotic protein kinase superfamily (29). S/T kinases have serine-threonine protein kinase catalytic domains and phosphorylate serine and threonine residues of target proteins. Important roles for S/T protein kinases in regulating cellular processes have been demonstrated in many eukaryotes (for a review, see references 19 and 81).

The number of S/T kinases in eukaryotic organisms is significant, with more than 100 genes in yeast, flies, and humans (57). Serine/threonine protein kinases (PKs) can be classified into groups based on their catalytic domains. The AGC (PKA, PKG, PKC) group includes PKC and the cyclic nucleotide-activated kinases PKA and PKG, the β-adrenergic receptor kinase (βARK) family, the ribosomal S6 kinase family, NDR (nuclear Dbf2-related) kinases, and other related families (29, 70). The CK1 (casein kinase 1, or cell kinase 1) group is a small but essential group of eukaryotic kinases that includes the CK1 protein family (29, 48). The CAMK (Ca^{2+}/calmodulin-dependent protein kinase) group contains the CAMK1 and CAMK2 protein kinase families but also contains several families of non-calcium-regulated kinases (29). STE (homologs of yeast sterile 7, 11, and 20) group kinases have been implicated in regulation of numerous mitogen-activated protein kinase (MAPK) pathways in various organisms (29, 37). The CMGC (CDK, MAPK, GSK3, CLK) group includes CDK (cyclin-dependent kinase), MAPK, GSK3 (glycogen synthase kinase 3), and CLK (CDK-like kinase) family kinases (29). The "Other" group includes many families (e.g., aurora kinase family [AUR], never-in-mitosis [NIMA]-related kinase family [NEK], polo-like kinase family [PLK], halotolerance family [HAL], Wee1 kinase family [WEE]) that are clearly eukaryotic protein kinases but cannot be easily classified into the other groups (57, 58). The "Atypical" group contains kinases that display little or no sequence similarity to eukaryotic protein kinase domains. Kinase families within this group include the histidine kinase family (HisK), phosphatidyl inositol 3′-kinase-related family (PIKK), pyruvate dehydrogenase kinase family (PDHK), and many others (57, 58). Kinases belonging to the same catalytic group are often functionally related, and accumulating studies indicate that multiple kinases can be implicated in a given cellular process (57). Therefore, a full understanding of the

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roles of S/T kinases cannot be accomplished without large-scale analysis. Since genome sequences are now available for a large number of organisms, systematic examination of all protein kinases in a species is more feasible.

The original annotation of the genome of the filamentous fungus *Neurospora crassa* predicted 89 S/T kinases (25). The most recent annotation predicts an additional 18 genes (Table 1, bottom), for a total of 107 genes (Table 1). Of the 107 S/T kinase genes, 32 have been previously analyzed (Table 1). The list includes the nine components of the three MAPK cascades (24, 42, 49, 53, 56, 68), PKA (5, 32), PKC (23), checkpoint kinase 2 (73), ATM (Ataxia telangiectasia mutated; *mus-21*) (87), ATR (ATM and Rad3-related) homologs (*mus-9*) (87), the NDR (nuclear Dbf2-related) family of S/T kinases, *cot-1* and *dbf-2* (20, 95), and others (see Table 1 for a complete list). Characterized *Neurospora* kinases have been shown to regulate a diversity of cellular functions, including polarized growth, hyphal fusion, asexual sporulation (conidiation), female sexual development, stress regulation, DNA damage responses, and the circadian clock (4, 18, 20, 22, 60, 68, 73, 84, 87, 95).

Chemical sensitivity screening has proven a robust method for identifying phenotypes for all gene deletion mutants in *Saccharomyces cerevisiae*, including those lacking S/T kinases (31). A proof-of-principle approach involved subjecting viable knockout mutants to sublethal concentrations of 12 chemicals, chosen to perturb a wide variety of cellular functions (69). Relative growth of mutants in the presence and absence of the chemicals was scored and compared relative to growth of the wild type. The data were then subjected to clustering analysis to allow grouping of chemicals with hypersensitive mutants. The results demonstrated known associations between gene products/pathways and chemicals, thus illustrating the viability of the approach. More importantly, this work revealed novel chemical interactions and pathways, including those with proteins of unknown function.

The *Neurospora* genome sequence is publicly available (25), and a large-scale gene knockout project for the ~10,000 predicted *Neurospora* genes is nearing completion (12, 13). We previously investigated the impact of transcriptional regulation on vegetative growth and asexual and sexual differentiation in *Neurospora* by examining 103 mutants lacking transcription factor genes (13). Here, we further exploit the gene knockout collection by investigating mutants lacking each of 86 S/T protein kinase genes in the *Neurospora* genome. We were unable to analyze three genes due to misannotation, and another 18 were predicted after the inception of this study. Morphological and growth phenotypes were analyzed (86), with 44 out of 77 viable knockout mutants displaying defects. Chemical sensitivity screens using a panel of eight treatments were introduced to augment identification of phenotypes. Using the latter approach, we were able to identify phenotypes for 25 viable mutants, bringing the total number of kinase genes with obvious functions to 61 in *Neurospora*. Finally, we demonstrate allelism between one kinase gene and a mapped morphological mutation.

MATERIALS AND METHODS

Strains and culture conditions. Vogel's minimal medium (VM) was used for vegetative growth and synthetic crossing medium (SCM) for development of female sexual structures (17). Sorbose-containing FGS medium was used for isolation of colonies on plates (17). Hygromycin (Calbiochem, San Diego, CA) was used at 200 μg/ml in media where indicated. Wild-type strains ORS-SL6a (FGSC 4200; *mat a*) and 74-OR23-IVA (FGSC 2489; *mat A*) were obtained from the Fungal Genetics Stock Center (FGSC; Kansas City, MO). Gene replacement mutants for S/T kinase genes were generated using a previously described high-throughput method (see Table S1 in the supplemental material) (13).

Mutant constructions. Deletion cassettes for kinase genes were constructed using yeast recombinational cloning in a 96-well plate system as described previously (12, 13). Cassettes were transformed into *Neurospora* mutants deficient in nonhomologous end-joining DNA repair (*mus-51* or *mus-52*) by electroporation in 96-well plates, with selection on hygromycin-containing FGS medium. Transformants were picked onto VM agar slants containing hygromycin and then crossed to wild-type 74-OR23-IVA (*mat A*) on SCM. Ejected ascospores were harvested from each cross and plated on FGS agar plates containing hygromycin. Spot testing was used to identify progeny that lacked the *mus-51* or *mus-52* mutation (both marked with *bar*, conferring resistance to phosphinothricin [2]) and to determine mating type. Homokaryotic mutant strains were confirmed by Southern blot analysis as described previously (13, 67).

In cases where ascospores of viable knockout mutants could not be isolated, attempts were made to isolate homokaryotic mutants in the vegetative phase by isolation of uninucleate microconidia (21) or by serial plating of macroconidia. In both of these cases, the resulting homokaryotic mutant strains retained the *mus-51* or *mus-52* mutant background. For microconidia isolation, transformants were inoculated on 0.1× SCM agar slants containing 0.5% sucrose and 1.0 mM sodium iodoacetate and incubated at 25°C under constant light for at least 10 days (21). Microconidia were harvested using 2.5 ml sterile water and then filtered through a Millex Durapore filter unit (5 μm; Millipore, Bedford, MA; microconidia pass through, while larger macroconidia are retained on the filter). Microconidia were collected by centrifugation, resuspended in sterile water, and plated on FGS solid medium containing hygromycin. For single spore isolation, macroconidia were harvested and plated onto FGS plates containing hygromycin. After incubation of plates at 30°C in the dark, a colony was picked and transferred to a VM-hygromycin slant. After growth for 5 days, macroconidia were isolated and plated onto a new FGS hygromycin plate, and a resistant colony was picked onto a fresh VM-hygromycin slant. This process was repeated 2 to 3 times. The presence of the gene deletion mutation for each kinase was confirmed by Southern blot analysis as described above (13).

We were unable to isolate homokaryotic mutants for nine genes because ascospores, microconidia, or serially transferred single spores carrying the gene replacement were not viable. Knockout cassettes for NCU11235, NCU06419, and NCU11410 were designed based on incorrect gene models later found to include a second gene (Table 1). As a result, two genes were disrupted in these strains, so the corresponding knockout mutants were excluded from the phenotypic analysis. The NCU09071 mutant was generated in a *his-3* background and was supplemented with histidine for phenotypic analysis.

Analysis of growth, morphological, and developmental phenotypes. Phenotypic analysis of each knockout mutant was performed by students in the *Neurospora* Genetics and Genomics Summer Research Institute (UCLA) (13, 86). Analysis of colony growth and morphology was performed using two different media at two different temperatures: VM and VM containing 2% yeast extract (VM+YE) at 25°C and 37°C. After inoculation, plates were incubated under ambient light/dark conditions in the laboratory. Hyphae at the colony edge and the entire colony were then photographed. Growth rates of basal hyphae were measured in glass race tubes containing VM agar medium at 25°C under ambient light/dark conditions over a 72-h period (86).

Slant tubes containing VM medium were inoculated with strains and grown at room temperature for 6 to 8 days. Production of conidia and aerial hyphae and overall pigmentation were then scored. Aerial hyphal extension was measured in VM or VM+YE standing liquid cultures. Test tubes containing 2 ml of liquid medium were inoculated and incubated statically at 25°C for 24 h. The top edge of the mycelial mat was marked on the tubes, and then the cultures were incubated for an additional 72 h. Total height (in mm) was recorded.

For analysis of female sexual fertility, mutants were inoculated on SCM plates containing 1.5% sucrose and incubated under ambient light/dark conditions at room temperature for 7 to 8 days. Cultures were scored for the formation of protoperithecia and then fertilized using wild-type conidia of the opposite mating type. Perithecial formation and ascospore development were scored 2 weeks after fertilization.

Trichogyne assays. Formation of trichogynes and chemotropic interactions between trichogynes and conidia of opposite mating type were analyzed as previously described (46). *Neurospora* wild-type (control) and mutant (ΔNCU02245) strains were grown on SCM agar for 6 days under constant light. A block of mycelia from the SCM plate was inoculated onto a 2% water-agar plate and incubated in a humid atmosphere at 25°C for 4 to 6 days under constant

TABLE 1. Summary of *Neurospora* serine-threonine protein kinase groups, families, and phenotypes

Group[a]	Family[b]	NCUno.[c]	*Neurospora* gene[d]	*S. cerevisiae* homolog[e]	Phenotype summary				
					Inviable	Growth of basal hyphae[f]	Asexual development[g]	Sexual development[h]	Chemical sensitivity[i]
AGC	AKT	03200	*stk-10*	*SCH9*		R	AH		SC
AGC	AKT	07280	*stk-50*	*YPK1/YPK2*	X	—[j]	—	—	—
AGC	NDR	03242	*stk-21*						
AGC	NDR	07296	*cot-1* (95)	*CBK1*		R			
AGC	NDR	07378	*stk-12*	*RIM15*			AH		
AGC	NDR	09071	*dbf-2* (20)	*DBF2/DBF20*		R	C, AH	PP, P, A	NS[k]
AGC	PDK1/PKA	03571	*stk-23*	*PKH1/PKH2*		R			T
AGC	PKA	00682	*pkac-2* (5)				AH		
AGC	PKA	06240	*pkac-1* (5)	*TPK1/TPK2/TPK3*		R	C, AH	PP, P	NS
AGC	PKC	06544	*pkc* (23)	*PKC1*	X	—	—	—	—
AGC	RSK	01797	*nrc-2* (49)	*KIN82/FPK1*		R	C, AH	P, A	NS
AGC	RSK/AKT	03197	*stk-11*	*YPK3*					
AGC	YANK	07062	*stk-49*						
CAMK	CAMK1	02283	*camk-2*						
CAMK	CAMK1	09123	*camk-1* (94)	*CMK1/CMK2*		R	C, AH	PP, P, A	NS
CAMK	CAMK1	09212	*camk-4*	*RCK1/RCK2*					B
CAMK	CAMKL	00914	*stk-16*	*FRK1/KIN4*		R		PP, P, A	B, SC, FL
CAMK	CAMKL	02245	*stk-19*	*YPL150W*				P, A	N/S
CAMK	CAMKL	04566	*prk-10*	*SNF1*					SC, T
CAMK	CAMKL	04747	*stk-31*	*KIN1/KIN2*		R	C, AH	PP, P, A	NS
CAMK	CAMKL	06249	*stk-40*	*PSK1/PSK2*					
CAMK	CAMKL	08346	*mus-58* (88)	*CHK1*			AH		SC
CAMK	CAMKL	09064	*stk-53*						
CAMK	CAMKL/CAMK unique	04143	*stk-26*	*PRR1*					
CAMK	RAD53	02751	*mus-59* (88)						
CAMK	RAD53	02814	*prd-4* (73)	*DUN1*					
CAMK	CAMK1/RAD53/CAMKL	06486	*stk-43*						
CK1	CK1	00685	*ck-1a* (27)	*HRR25*	X	—	—	—	—
CK1	CK1	04005	*ck-1b* (93)	*YCK1/YCK2/YCK3*	X	—	—	—	—
CMGC	CDK	01435	*stk-1*		X	—	—	—	—
CMGC	CDK	03659	*prk-3*	*KIN28*					T
CMGC	CDK	04426	*div-4*	*CAK1*		R		PP, P, A	M, NS
CMGC	CDK	06685	*stk-47*	*CTK1*		R		PP, P, A	C
CMGC	CDK	07172	*stk-8*	*SSN3*		R	C, AH	PP, P, A	NS
CMGC	CDK	07580	*mdk-1*	*PHO85*	X	—	—	—	—
CMGC	CDK	07880	*prk-6*						
CMGC	CK2	03124	*cka* (60)	*CKA1/CKA2*			AH		SC
CMGC	CLK	00230	*prk-4*	*KNS1*	X	—	—	—	
CMGC	DYRK	06638	*stk-46*			R		PP, P, A	M
CMGC	DYRK	07872	*prk-2*	*YAK1*		R	C, AH	PP, P, A	NS
CMGC	DYRK	10853	*stk-57*						
CMGC	GSK	04185	*gsk-3* (20)	*RIM11/MRK1*					B
CMGC	MAPK	02393	*mak-2* (53)	*FUS3/KSS1*		R	C, AH	PP, P, A	NS
CMGC	MAPK	07024	*os-2* (92)	*HOG1*			C,	PP, P, A	NS
CMGC	MAPK	09842	*mak-1* (56, 68)	*SLT2*		R	C, AH	PP, P, A	N/S
CMGC	RCK	01498	*ime-2* (38)	*IME2*		R	AH		F
CMGC	SRPK	09202	*mdk-2*	*SKY1*		R	C, AH	PP, P, A	NS
CMGC	CLK/SRPK	05655	*stk-35*			R	AH	PP, P, A	NS
CMGC	CLK/SRPK	05658	*stk-36*			R	C, AH	PP, P, A	NS
CMGC	CLK/SRPK/DYRK	09189	*stk-54*						C
CMGC	CLK/SRPK	10004	*stk-56*						
Other	AUR	00108	*stk-13*	*IPL1*	X	—	—	—	—
Other	CAMKK	03523	*stk-22*	*TOS3/SAK1*		R	AH		C, F
Other	CAMKK	06177	*camk-3*						
Other	CDC7	11410	*cdc7*	*CDC7*	NA[l]	NA	NA	NA	NA
Other	HAL	01940	*ptk-2* (52)	*PTK1/PTK2*		R			F
Other	HAL	04335	*stk-30*	*NPR1/PRR2*					SC
Other	HAL	06179	*stk-5*	*SAT4*					SC
Other	IRE	02202	*stk-14*	*IRE1*					
Other	NAK	06202	*stk-38*	*ARK1/PRK1*		R	C, AH	PP, P	C, B, M
Other	NAK	07399	*stk-9*	*YPL236CP*			C, AH	PP, P, A	F, T, M
Other	NEK	03187	*nim-1* (75)	*KIN3*				A	B
Other	PEK	01187	*cpc-3* (78)	*GCN2*		R			
Other	PLK	09258	*cdc5*	*CDC5*	X	—	—	—	—
Other	RAN	04990	*stk-17/fi* (59)	*SKS1/VHS1*		R	C, AH	PP, P, A	T
Other	RAN	06230	*stk-39*						

Continued on following page

TABLE 1—*Continued*

Group[a]	Family[b]	NCUno.[c]	*Neurospora* gene[d]	*S. cerevisiae* homolog[e]	Phenotype summary				
					Inviable	Growth of basal hyphae[f]	Asexual development[g]	Sexual development[h]	Chemical sensitivity[i]
Other	SCYI	04755	*stk-32*	*SCY1*					
Other	ULK	00188	*apg-1*	*ATG1*		R	AH	PP, P, A	F, S, SC, T, M
Other	VPS15	06626	*stk-45*	*VPS15*					
Other	WEE	04326	*stk-29*	*SWE1*					B
Other	IKS	08177	*stk-51*	*IKS1*					
STE	STE11	01335	*cdc15*					PP, P, A	
STE	STE11	02234	*mik-1* (68)	*BCK1*		R	C, AH	PP, P, A	NS
STE	STE11	03071	*os-4* (24, 42)	*SSK2/SSK22*			C,	PP, P, A	NS
STE	STE11	06182	*nrc-1* (49)	*STE11*		R	C, AH	PP, P, A	NS
STE	STE20	00406	*vel* (this study)	*CLA4/SKM1*		R	C, AH	PP, A	NS
STE	STE20	00772	*mst-1* (20)					A	
STE	STE20	03894	*stk-4*	*STE20*		R			T, M
STE	STE20	04096	*prk-9*			R	C, AH	PP, P, A	NS
STE	STE20	11235	*pod-6* (79)		NA	NA	NA	NA	NA
STE	STE7	00587	*os-5* (24)	*PBS2*			C,	PP, P, A	NS
STE	STE7	04612	*mek-2* (56)	*STE7*		R	C, AH	PP, P, A	NS
STE	STE7	06419	*mek-1* (68)	*MKK1/MKK2*	NA	NA	NA	NA	NA
Unclassified		02885	*stk-20*						
Unclassified		05638	*stk-34*						
Unclassified		06006	*stk-37*						
Unclassified		06421	*stk-41*						
Unclassified		06422	*stk-42*						
Unclassified		06583	*stk-44*						
CMGC	CDK	09778	*cdc28*	*CDC28*					
Other	BUD32	04595	*prk-11*	*BUD32*					
Other	SCY1	04279	*stk-28*	*CEX1*					
Atypical	ABC1	03823	*stk-25*	*ABC1*					
Atypical	ABC1	04259	*stk-27*	*YLR253W*					
Atypical	ABC1	05600	*stk-33*	*YPL109C*					
Atypical	BRD	09595	*stk-55*						
Atypical	PDHK	03796	*stk-24*	*YGL059W*					
Atypical	PDHK	06760	*stk-48*						
Atypical	PDHK	11744	*stk-58*	*PKP1*					
Atypical	PIKK	00274	*mus-21* (87)	*TEL1*					
Atypical	PIKK	01379	*stk-18*	*TRA1*					
Atypical	PIKK	05608	*div-18*	*TOR1/TOR2*					
Atypical	PIKK	11188	*mus-9* (87)	*MEC1*					
Atypical	RIO	07722	*rgb-40*	*RIO2*					
Atypical	RIO	08767	*stk-52*	*RIO1*					
Atypical	TAF1	02556	*hat-2*	*TAF1*					
TKL	LRRK	05808	*tkl-1*						

[a] Group abbreviations: AGC, PKA (protein kinase A/cyclic AMP-dependent protein kinase), PKG (protein kinase G/cGMP-dependent protein kinase), PKC (protein Kinase C); CK1 (casein kinase 1 or cell kinase 1); CAMK (Ca^{2+}/calmodulin-dependent protein kinase); CMGC, cyclin-dependent, mitogen-activated, glycogen synthase and cyclin-dependent protein kinase-like kinases; STE, sterile; TKL, tyrosine kinase-like kinase.

[b] Family abbreviations: AKT, oncogene protein of v-akt; NDR, nuclear Dbf2 related; PDK1, phosphoinoside-dependent protein kinase; PKA, protein kinase A or cyclic AMP-dependent protein kinase; PKC, protein kinase C; RSK, ribosomal s6 kinase; YANK, yet another novel kinase; CAMK, Ca^{2+}/calmodulin-dependent protein kinase; CAMKL, Ca^{2+}/calmodulin-dependent protein kinase-like kinase; RAD, radiation sensitive; CK, casein kinase or cell kinase; CDK, cyclin-dependent protein kinase; CLK, cyclin-dependent protein kinase-like kinase; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; GSK, glycogen synthase kinase; MAPK, mitogen-activated protein kinase; RCK, related to murine RCK (*ros* cross-hybridizing kinase); SRPK, serine-rich protein kinase; AUR, Aurora; CAMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; CDC, cell division cycle; HAL, halotolerance; IRE, inositol-requiring protein; NAK, NF-κB-activating kinase; NEK, NIMA-related kinase; PEK, pancreatic alpha-subunit of eukaryotic initiation factor kinase; PLK, polo kinase; RAN, Ran GTPase kinase; SCY1, related to *S. cerevisiae* Scy1 kinase; ULK, Unc-51-like kinase; VPS, vacuolar protein sorting; WEE, small; IKS, Ira1 kinase suppressor; STE, sterile; ABC1, related to *S. cerevisiae* Abc1 kinase; BRD, bromodomain; PDHK, pyruvate dehydrogenase kinase; PIKK, phosphatidyl inositol 3′-kinase-related kinase; RIO, right open reading frame; TAF1, TATA-binding protein-associated factor 1; LRRK, leucine-rich repeat kinase.

[c] Based on version 5 annotation of the Broad Institute's *Neurospora crassa* database (www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html).

[d] References for previously studied genes are in parentheses. Kinase names are consistent with the *Neurospora* e-Compendium Project at Leeds University (http://bmbpcu36.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm). *stk-18* through *stk-58* were named during this study, in accordance with the e-Compendium system.

[e] Yeast orthologs were obtained from the literature or assigned during this study based upon analyses of phylogenetic trees available at www.phylomedb.org (34, 35).

[f] R, reduced growth.

[g] Asexual phenotypes are represented by phenotypes in aerial hyphae (AH) or conidial development (C).

[h] The sexual phenotypes are represented by their occurrence during protoperithecial (PP), perithecial (P), or ascospore (A) development.

[i] Chemical phenotypes are summarized based on sensitivity or resistance to cytochalasin A (C), benomyl (B), FK-506 (F), sorbitol (S), sodium chloride (SC), fludioxonil (FL), *tert*-butyl hydroperoxide (T), and menadione (M).

[j] —, phenotypic assay could not be performed due to inviability of knockout mutant.

[k] NS, mutant was not analyzed by chemical screening due to poor growth.

[l] NA, mutant was not analyzed for phenotypes due to simultaneous mutation of adjacent gene.

light. The plates were transferred to ambient humidity conditions and incubated 3 to 5 more days. A small square block (0.5 by 0.5 cm) of thin water agar was placed on the water agar plate in a region covering a few protoperithecia. A volume containing 1 μl of a wild-type microconidial suspension was placed on the water agar block. Migration of trichogynes into the block was examined after 16 h and then at 24-h intervals using an Olympus BX41 compound microscope (Olympus America, Lake Success, NY) with UM Plan fluorite objective lenses.

Chemical sensitivity screening. All viable S/T kinase knockout mutants were screened for responses to the reactive oxygen species (ROS)-generating agent menadione. Race tubes containing 1× VM salts, 1.5% sucrose, 50 ng/ml biotin, and 1.5% agar with or without 100 μM menadione (M5750; Sigma) were inoculated with each mutant. Tubes were incubated in constant light at 25°C for 24 h before transfer to constant darkness at 25°C, after which time the growth front was marked every 24 h. Growth rates were measured and assigned to a growth rate range (±2.5 mm/day) estimated over 4 days. The growth rate in the presence of menadione was normalized to that on medium lacking menadione.

Cytochalasin A (40 ng/ml; Sigma, St. Louis, MO), benomyl (92 ng/ml; Fluka, St. Louis, MO), FK-506 (50 ng/ml; LC Laboratories, Woburn, MA), *tert*-butyl hydroperoxide (0.13 mM; Sigma, St. Louis, MO), sorbitol (0.8 M; Sigma), sodium chloride (0.35 M; EMD Chemicals, Gibbstown, NJ), and fludioxonil (2.75 ng/ml) were used for chemical screens of viable S/T kinase mutants with growth rates at least 50% of wild type on VM. The concentration of each chemical used was determined as the amount that inhibited wild-type growth by ~70%. VM agar plates with or without chemical were inoculated with wild-type and knockout strains in quadruplicate. Plates were incubated in constant darkness at 30°C for 22 to 24 h. Colony radii were measured, and the percent growth in the presence versus absence of the chemical was calculated for each of the four measurements. Data from three independent experiments (four measurements/experiment) were subjected to a Q test to remove outliers. Data were then analyzed using a *t* test. Mutants were considered sensitive or resistant if they displayed results within at least 95% confidence in two out of three trials and at least 80% confidence in all three trials.

Complementation of ΔNCU00406 and *velvet* mutants. To test for a possible allelic relationship between NCU00406 and the *velvet* (*vel*) mutation, a construct containing the NCU00406 open reading frame (ORF) and promoter region was generated and transformed into *vel* and ΔNCU00406 mutants. A fragment containing the NCU00406 ORF, as well as 3 kb upstream and 0.65 kb downstream (total size, ~6 kb), was amplified by PCR using primers cla45F (5′-TTGAGAG CTCGCAGTTGGTAGGAACACA-3′) and cla43R (5′-CTTCTCTAGACTCC TCCTGATTCGTTGA-3′) and digested with SacI and XbaI. The fragment was then ligated into pTJK1 digested with SacI and XbaI to yield pGP7. pTJK1 contains the *bar* dominant selectable marker, conferring resistance to phosphinothricin (42). pGP7 was then transformed into conidia from the ΔNCU00406 and *vel* mutants by using electroporation as described previously (40). Transformants were selected on FGS solid medium containing phosphinothricin (66). Homokaryotic strains were isolated by serial plating of macroconidia as described above and then confirmed by Southern blot analysis (67).

RESULTS

Serine/threonine protein kinase genes in the *Neurospora* genome. Currently, 107 serine/threonine protein kinase genes are predicted in *N. crassa*. At the inception of this study, 89 S/T kinases were annotated and selected for analysis. To classify each of the S/T kinases to a specific group (36, 58), protein sequences were submitted as a query for a BLAST algorithm at the Salk Institute's Protein Kinases, Kinomes and Evolution website, using a database of protein kinase genes. Among the 89 genes in our study, 6 appeared to be unique to filamentous fungi, while the remaining 83 could be categorized into the following groups: AGC, CAMK, CK1, CMGC, Other, and STE (Table 1).

To compare S/T kinases across diverse eukaryotic phyla, kinases from human, plant (*Selaginella moellendorffii*), yeast (*S. cerevisiae*), and *Neurospora crassa* were analyzed (Table 2). The distributions of kinases among the AGC, Atypical, CAMK, CK1, CMGC, Other, and STE groups were compared. While additional kinases belonging to other groups (e.g., tyrosine kinases and histidine kinases) are known for each of these organisms, the 83 *Neurospora* kinases with homology to proteins outside the filamentous fungi can be classified within six of the seven groups above. The Atypical kinases were annotated after the inception of this study. While specific kinases within the Atypical group are known to be S/T kinases (87), it is unclear whether all kinases in this group phosphorylate serine and threonine residues. We therefore focused our analyses on the AGC, CAMK, CK1, CMGC, Other, and STE groups. *Neurospora* has the fewest kinases among the organisms analyzed in Table 2. This is likely caused by the phenomenon of RIP (repeat-induced point mutation), where one copy of a duplicate sequence is detected and mutated (80). This has resulted in a streamlined genome containing fewer paralogs than most eukaryotic organisms (25, 26). This lack of redundancy makes *Neurospora* an ideal organism in which to study the function of kinases. Correcting for differences in the total number of kinases, relative kinase group sizes are essentially uniform across all of the organisms in our comparison. As expected, the relative group sizes of human, yeast, and *Neurospora* are slightly more similar to one another than they are to those in plants. *Neurospora* also contains members of protein kinase families and subfamilies that are known to be fungal specific (57). *Neurospora* S/T kinase gene names were taken from the literature, the e-compendium at Leeds University (http://bmbpcu36.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm), or assigned during this study (Table 1). *S. cerevisiae* orthologs were taken from the literature or assigned during this study based upon analyses of phylogenetic trees available at www.phylomedb.org (Table 1) (33, 34).

TABLE 2. Classification of serine-threonine protein kinases[a] in human, plant, yeast, and *Neurospora crassa*

Group	No. of serine-threonine kinases from the group in:			
	Human	Plant[b]	Yeast[c]	*N. crassa*
AGC	63	33	17	13
Atypical[d]	44	33	12	14
CAMK	74	139	22	14
CK1	12	9	4	2
CMGC	64	93	23	23
Other	81	76	38	22
STE	47	36	14	12

[a] Additional kinases and groups comprise the kinomes of each organism.
[b] *S. moellendorffii*.
[c] *S. cerevisiae*.
[d] Histidine kinases were omitted from the Atypical group.

We attempted gene replacement of the 89 S/T protein kinase genes as part of the *Neurospora* Genome Project. In three cases (NCU06419, NCU11235, and NCU11410), the annotation suggests that adjacent genes were also mutated. Therefore, these three genes were excluded from further study (Table 1) (see Materials and Methods). We were able to generate homokaryotic knockout mutants for 49 genes as ascospore progeny from sexual crosses. We obtained homokaryotic mutants for another 28 kinase genes through isolation of uninucleate microconidia or after serial transfer of macroconidia. We were unable to isolate viable knockout mutants for the remaining nine kinase genes (Table 1). This left us with a total of 77 mutants for phenotypic analysis.

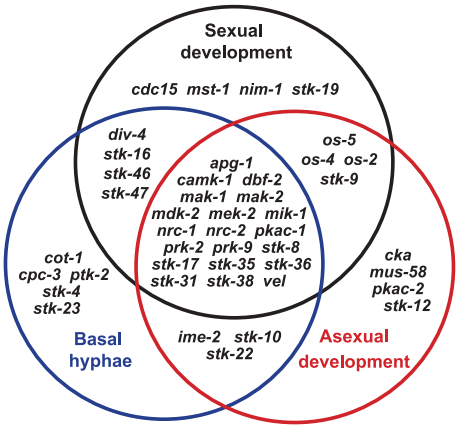

FIG. 1. Venn diagram showing distribution of S/T protein kinase mutants with phenotypes. The 44 viable mutants exhibiting defects in at least one of the three major growth/developmental pathways are indicated as gene names for the deleted genes.

A majority of the viable S/T protein kinase mutants exhibit growth or developmental phenotypes. We analyzed vegetative hyphal growth, asexual development, and sexual development in the 77 viable knockout mutants (13, 67). *Neurospora* grows vegetatively by apical extension, branching, and fusion of basal hyphae. Under the condition of nutrient deprivation or an air-water interface, *Neurospora* enters the developmental program for production of multinucleate asexual spores, macroconidia. Initially, aerial hyphae are differentiated from basal (vegetative) hyphae. These aerial hyphae then begin a budding routine at their tips, to form conidiophores which ultimately give rise to macroconidia (82). Sexual development is induced by nitrogen starvation in *Neurospora*, with differentiation of female reproductive structures (protoperithecia) (76). Fertilization is accomplished by chemotropic growth of a specialized female hypha (trichogyne) toward a male cell of opposite mating type, transport of the male nucleus into the protoperithecium, mitosis, and cell proliferation, followed by nuclear fusion. Meiosis ensues, and the protoperithecium develops into the mature fruiting body (perithecium) containing the meiotic progeny (ascospores) (76).

Our analysis revealed that 32 and 31 kinase mutants had abnormalities in basal hyphal growth and asexual development, respectively (Fig. 1 and Table 1; see also Table S1 in the supplemental material for detailed phenotypic data). Among these, 23 mutants were defective in both hyphal growth and asexual development (Fig. 1 and Table 1). Mutants lacking NDR family kinase *cot-1*, PDK1/PKA family member *stk-23*, HAL (halotolerance) family *ptk-2*, PEK (pancreatic eIF-2α kinase) family member *cpc-3*, and STE20 family member *stk-4* were distinctive in that their only morphological phenotype was a defect in hyphal growth. Interestingly, the *stk-4* ortholog *ste20* is not only required for normal growth in *S. cerevisiae* but is also essential for mating and pseudohyphal differentiation (99). In contrast to the *Neurospora* genes specific for hyphal growth, NDR family mutant Δ*stk-12*, PKA family mutant Δ*pkac-2*, CAMKL (Ca^{2+}/calmodulin-dependent protein kinase-like) family mutant Δ*mus-58*, and CK2 (casein kinase 2) family mutant Δ*cka* only possessed defects in asexual sporulation. *S. cerevisiae* does not possess an asexual sporulation pathway; however, loss of the *stk-12* ortholog in *S. cerevisiae* leads to defects in sexual sporulation, along with other phenotypes (83).

A large proportion of S/T kinase mutants displayed defects in sexual development. The genes replaced in STE20 family Δ*mst-1*, STE11 family Δ*cdc15*, CAMKL family Δ*stk-19*, and NEK family Δ*nim-1* appear to be specific for sexual development, while the majority of genes implicated in sexual development also play roles during asexual growth and development (Fig. 1). Most of the sexual developmental mutants were not able to produce protoperithecia (Table 1; see also Table S1 in the supplemental material). The CAMKL family mutants Δ*stk-16* and Δ*stk-31* and the CDK family mutant Δ*div-4* formed protoperithecia but had defects in the timing or number of female structures produced. Three mutants formed normal perithecia but ejected no (Δ*nim-1*), few (Δ*mst-1*), or white ascospores (Δ*vel*; 20% of ascospores were white.). One of the *S. cerevisiae vel* homologs, *cla4*, is required for normal pheromone sensitivity (10, 41). This suggests that Cla4p acts at an earlier point in sexual development in yeast than VEL in *Neurospora*.

Most of the 32 mutants that exhibited abnormalities in sexual development also possessed defects in basal hyphae growth and asexual differentiation (Table 1). Among these mutants are those lacking components of the MIK-1/MEK-1/MAK-1 and NRC-1/MEK-2/MAK-2 MAPK pathways. The MAK-1 pathway is required for normal growth, hyphal fusion, conidiation, differentiation of protoperithecia, and cell wall integrity (56, 68), while the MAK-2 cascade controls growth, hyphal fusion, conidiation, and protoperithecial development (49, 53, 56). Strains with mutations in the proteins of the osmotic stress resistance MAPK pathway (OS-4/OS-5/OS-2) have near-normal growth rates but exhibit defects in conidiation and sexual development while displaying sensitivity to osmotic stress and resistance to phenylpyrrole and dicarboximide fungicides (24, 42, 92). The roles for these MAPK pathways in *Neurospora* share many parallels with the corresponding pathways in *S. cerevisiae* and other fungi. For example, in *S. cerevisiae*, the MAK-2 homologous pathway is required for the pheromone response and filamentous growth, the MAK-1-related pathway is necessary for cell wall integrity and mitotic progression, and the OS-2 homologous MAPK pathway is required for resistance to osmotic stress (11).

The Δ*stk-19* mutant was of particular interest, since it formed protoperithecia normally but produced very few perithecia after fertilization. We have previously observed such a phenotype in mutants with defects in chemotropic growth of female-specific hyphae, or trichogynes, toward male cells (conidia) (46). In order to determine whether Δ*stk-19* possessed such a chemotropism defect, we further investigated this mutant in a trichogyne chemotropism assay (46) (Fig. 2). The results showed that although many Δ*stk-19* protoperithecia form trichogynes, they are largely unable to recognize or encircle the male conidia (Fig. 2). This result suggests that the missing kinase is required for chemotropism of females toward males during fertilization. In *S. cerevisiae*, the *stk-19* ortholog YPL150W is required for a normal growth rate and glycogen accumulation but has no known roles in mating or sexual development (91, 99).

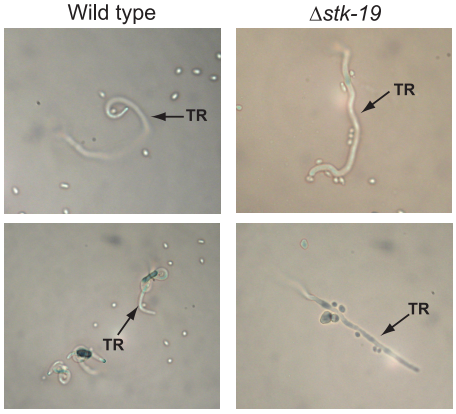

FIG. 2. Trichogyne chemotropism in the mutant lacking NCU02245/*stk-19*. The wild type and the ΔNCU02245 (Δ*stk-19*) strain were cultured to produce female reproductive structures (protoperithecia). Sterile agar blocks were then placed on top of protoperithecia. Subsequently, a conidial suspension (males) from a wild-type strain of opposite mating type was applied to the top of the agar block. Trichogyne (TR) growth and migration through the agar blocks toward the males was monitored microscopically 16 to 24 h after application of the conidia.

Overall, our results showed that 44/77 mutants analyzed (57%) exhibit a defect in at least one of the three major phenotypes analyzed (Fig. 1; Table 1). Among these, 11 and 20 knockout mutants possess defects in two or three traits, respectively (Fig. 1; Table 1). The number of genes implicated in multiple traits (31) in combination with those for which no viable mutant could be isolated (9) suggest that a large proportion of *Neurospora* S/T kinases play crucial roles in important fungal life processes (Fig. 1).

Chemical sensitivity assays increase the number of S/T protein kinase mutants with phenotypes. In *S. cerevisiae*, most genes that do not exhibit obvious functions during growth and development are essential for normal resistance to at least one chemical treatment (31). In order to better understand the functions of S/T kinases in *Neurospora*, we compared mutants to the wild type with regard to their sensitivity to a panel of chemical treatments (see Table 1 and the detailed chemical phenotypes in Table 3). The chemicals included those that induce oxidative (menadione and *tert*-butyl hydroperoxide) or osmotic (sorbitol and sodium chloride) stress, perturb the cytoskeleton (benomyl and cytochalasin A), act as a fungicide (fludioxonil), or inhibit the Ca^{2+}-dependent phosphatase calcineurin (FK-506). In order to avoid issues with slow-growing strains that may have skewed our results, we included in our analysis only those viable mutants (56) that had growth rates of at least 50% of the wild type on normal medium.

Menadione generates a hyperoxidative condition by producing ROS, while *tert*-butyl hydrogen peroxide is a source of the oxidant peroxide (6, 44). Three mutants, ULK (Unc-51-like kinase) family Δ*apg-1*, STE20 family Δ*stk-4*, and NAK (NF-κB activating kinase) family Δ*stk-9*, were hypersensitive to both menadione and *tert*-butyl hydrogen peroxide (Table 3), sug-

TABLE 3. Chemical sensitivity summary

Deleted gene	Sensitivity[a]							
	Cytochalasin A	Benomyl	FK506	Sorbitol	Sodium chloride	Fludioxonil	*tert*-Butyl hydroperoxide	Menadione
stk-10					S			
stk-23							S	
camk-4		R						
stk-16		S			S	S		
prk-10					R		S	
mus-58					S			
prk-3							S	
div-4								S
stk-47	S							
cka					S			
stk-46								S
gsk-3		R						
ime-2			R					
stk-54	R							
stk-22	R		R					
ptk-2			S					
stk-30					S			
stk-5					S			
stk-38	S	S						S
stk-9			S				S	S
nim-1		R						
stk-17/fi							S	
apg-1			S	S	S		S	S
stk-29		S						
stk-4							S	S

[a] Mutants were classified as sensitive (S) or resistant (R) relative to the wild type based on the percentage of growth in the presence of the chemical compared to growth in the absence of the chemical, as follows: percent growth = (colony radius with chemical)/(colony radius without chemical) × 100. See Materials and Methods for details. For all compounds, sensitivity was scored directly from the average growth rate, except for menadione, for which sensitivity was scored after the average growth rates were binned (see Table S1 in the supplemental material). Corroboration of findings was based on the version 5 annotation of the Broad Institute's *Neurospora crassa* database (www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html).

gesting the missing kinases play general roles in resistance to oxidative stress. Another three and four mutants were more sensitive than the wild type to menadione or *tert*-butyl peroxide, respectively (Table 3). With a total of seven sensitive mutants, *tert*-butyl peroxide yielded the greatest number of phenotypes in our study. Reactive oxygen species have previously been implicated in control of conidiation in *Neurospora* (1). Therefore, it is of interest that four mutants (Δ*stk-38*, Δ*stk-9*, Δ*stk-17/fi*, and Δ*apg-1*) all possess defects in some aspect of conidiation, in addition to altered sensitivity to *tert*-butyl peroxide and/or menadione.

We assayed the response of kinase mutants to two agents that induce osmotic stress in fungi, sorbitol and sodium chloride. One mutant (Δ*apg-1*) that was sensitive to menadione and peroxide also displayed decreased growth in the presence of sorbitol and sodium chloride, consistent with a general sensitivity to environmental stress. In *S. cerevisiae*, the *apg-1* homologous gene, *atg1*, is required for autophagy, regulation of cellular ROS levels, and sensitivity to the complex chemical propolis but has no reported roles in resistance to osmotic stress (101). In total, seven *Neurospora* mutants exhibited significant sensitivity to sodium chloride, while one displayed increased resistance (Table 3). Of the sensitive mutants, two (Δ*stk-5* and Δ*stk-30*) lack genes of the HAL (halotolerance) family. This result is a significant proof of principle, as the HAL family is known to function in salt tolerance in *S. cerevisiae* (63).

To explore the role of S/T kinases in cytoskeletal maintenance, the mutants were screened using two chemicals known to alter two major cytoskeletal components. Cytochalasin A interacts with actin filaments and inhibits their polymerization and elongation (14). Benomyl binds to microtubules, resulting in the inhibition of crucial cellular processes such as mitosis, meiosis, and cellular transport (90). The growth of two kinase mutants, Δ*stk-38* (NAK family) and Δ*stk-47* (CDK family), was decreased more than the wild type in the presence of cytochalasin A. Two mutants displayed increased resistance relative to the wild type, Δ*stk-22* (CAMKK [Ca^{2+}/calmodulin-dependent protein kinase kinase] family) and Δ*stk-54* (CLK/SRPK [serine-rich protein kinase]/DYRK [dual-specificity tyrosine-regulated kinase] family) (Table 3). Three kinase mutants (Δ*nim-1* and Δ*gsk-3* [GSK family] and Δ*camk-4* [CAMK1 family]) were more resistant to benomyl, while three mutants (Δ*stk-16* [CAMKL family], Δ*stk-29* [WEE family], and Δ*stk-38* [NAK family]) exhibited greater sensitivity (Table 3). Notably, mutant Δ*stk-38* exhibited significant sensitivity to both cytochalasin A and benomyl (Table 3). The observation of altered sensitivity of a mutant to cytochalasin A and/or benomyl is consistent with a defect in the actin and/or β-tubulin cytoskeleton, respectively. Four mutants, Δ*stk-16*, Δ*stk-22*, Δ*stk-38*, and Δ*stk-47*, exhibited defects during vegetative or sexual development in the absence of chemicals, possibly resulting from disturbance of the cytoskeleton. Interestingly, one of the *S. cerevisiae stk-16* orthologs, *KIN4*, inhibits the mitotic exit network and is localized to the mother cell cortex, spindle pole bodies, and bud neck (16, 71). Additionally, the *S. cerevisiae* orthologs of *stk-38*, *ARK1* and *PRK1*, are important for regulation of the cortical actin cytoskeleton (15). Deletion of *ARK-1* results in formation of actin clumps and a growth defect in *S. cerevisiae*. Of interest, the *Neurospora* Δ*stk-38* mutant exhibits defects in hyphal growth and asexual and sexual development. Finally, while the sensitivity of the *Neurospora* Δ*stk-47* mutant to cytochalasin A suggests an influence on actin organization, the *S. cerevisiae* ortholog *CTK1* may play a role in microtubule maintenance, given the sensitivity of the yeast *ctk1* mutant to benomyl (69).

FK-506 is an immunosuppressant drug that inhibits calcineurin, an S/T protein phosphatase in the calcium signaling pathway (74). Three S/T kinase mutants, Δ*apg-1*, Δ*ptk-2*, and Δ*stk-9* (NAK family), were more sensitive to FK-506 than the wild type, while two mutants, Δ*ime-2* (RCK [*ros* cross-hybridizing kinase] family) and Δ*stk-22* (CAMKK family) were more resistant to FK-506 (Table 3). Altered sensitivity to FK-506 implicates these kinases in calcineurin function in *Neurospora*. The finding that the Δ*ime-2* mutant has altered sensitivity to FK-506 is a novel finding in any fungal species. Work in the Glass laboratory has shown that *ime-2* is not required for meiosis, as in *S. cerevisiae*, but instead negatively regulates formation of female reproductive structures in response to adequate nitrogen in *Neurospora* (38). This function is similar to that described for the filamentous fungus *Cryptococcus neoformans* (39, 55). In our study, we did not screen for protoperithecial formation under nitrogen-rich conditions. However, we did note that *ime-2* mutants had defects in hyphal growth and aerial hyphae formation, in addition to altered sensitivity to FK-506.

Fludioxonil is a member of the phenylpyrrole class of fungicides (64). Fludioxonil inappropriately stimulates the *osmotic sensitive-2* (OS-2) MAPK pathway, leading to excessive glycerol production and subsequent cell death in filamentous fungi (100). All *Neurospora os* mutants, including those with defects in the three genes of the *os* MAPK pathway, are resistant to fludioxonil (24, 100). In accordance with previous results, we observed that the three *os* MAPK pathway mutants were resistant to fludioxonil but sensitive to sorbitol and sodium chloride (data not shown). In addition, we noted another previously uncharacterized S/T kinase mutant (Δ*stk-16*; CAMKL family) that was sensitive to both fludioxonil and sodium chloride, as well as benomyl (Table 3). This mutant is of interest, as it appears to have uncoupled the previously observed association between fungicide resistance and osmotic sensitivity and may participate in a novel pathway that modulates fungicide resistance in *Neurospora*. Δ*stk-16* also possesses defects in hyphal growth and protoperithecial development (Table 1). One of the homologous genes in *S. cerevisiae*, *KIN4*, modulates mitotic exit but has not been implicated in osmotolerance (16).

Taken together, the results revealed 25 mutants with chemical sensitivity phenotypes. Accounting for mutants that exhibited a significant sensitivity or resistance to more than one chemical, a total of 38 chemical phenotypes were observed. Importantly, these screens uncovered unique phenotypes for eight mutants that did not exhibit any morphological or developmental defects. This corresponds to more than 10% of the viable S/T kinase mutants and brings the total number of viable mutants with phenotypes to 52.

NCU00406 is allelic with *VELVET*, encoding a PAK with homology to Cla4p and Skm1p from *S. cerevisiae*. While performing phenotypic analysis, we observed that the ΔNCU00406 mutant possessed severe defects in extension of basal and aerial hyphae and a distinctive colony morphology.

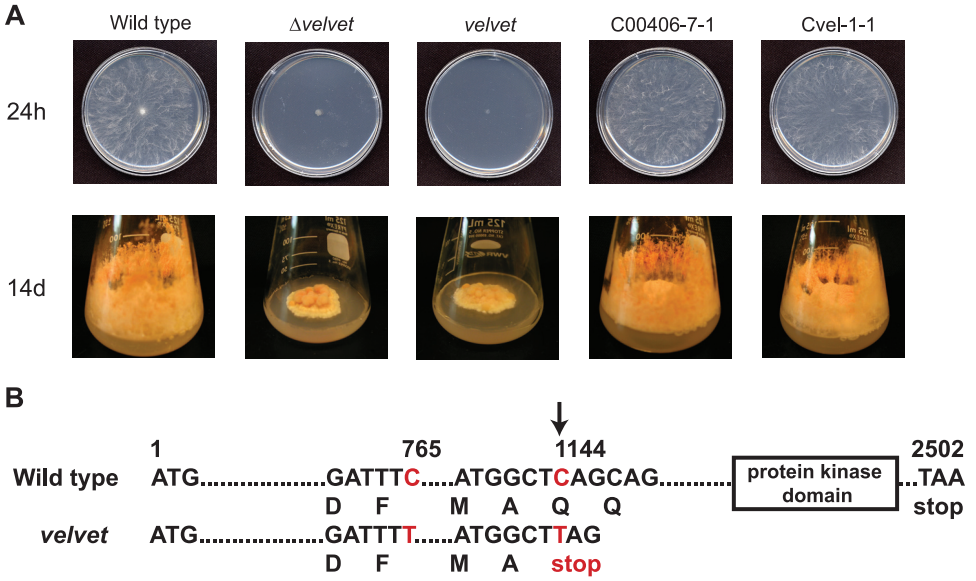

FIG. 3. Allelism between the S/T kinase gene NCU00406 and *velvet* (*vel*). (A) Colony morphology and vegetative growth of ΔNCU00406 and *vel* mutants complemented with a construct containing the wild-type NCU00406 gene. C00406-7-1 and Cvel-1-1 are ΔNCU00406 and *vel* mutants, respectively, transformed with the complementation construct for NCU00406. Strains were grown in VM agar plates (top) and flasks (bottom) for 24 h and 14 days, respectively. (B) Sequence analysis of the NCU00406 ORF region in the *vel* mutant. After amplification and cloning of the NCU00406 ORF region from the *vel* mutant, the 2,502-bp sequence was compared with the corresponding sequence from the wild type. The synonymous mutation at position 765 that does not alter the amino acid sequence is shown, along with the premature stop codon at 1144.

The mutant was yellow, with aggregated hyphae and little production of conidia. We noted that the phenotype of ΔNCU00406 resembled that of the morphological mutant *velvet* (*vel*), isolated more than 50 years ago (72). Furthermore, the *vel* mutation maps near cloned genetic markers in the vicinity of NCU00406.

Based on the above information, we investigated whether *vel* is allelic with NCU00406. We transformed *vel* and the ΔNCU00406 mutants with a construct containing the NCU00406 open reading frame and promoter region. In both cases, introduction of a wild-type allele of NCU00406 complemented growth and morphological phenotypes (Fig. 3A). In order to determine the position of the mutation(s) in *vel*, we amplified regions of the NCU00406 gene from the *vel* and wild-type strains and submitted them for sequencing. The results revealed two single base pair substitutions, a C-to-T change at nucleotide position 765 and another C-to-T change at position 1144 in the 2,502-bp ORF of the *vel* allele. The substitution at position 765 is synonymous, retaining the codon for phenylalanine. However, the second substitution produces a nonsense mutation at nucleotide position 1144, predicted to result in a truncated protein lacking the protein kinase domain (Fig. 3B). Taken together, these results demonstrate that *vel* is allelic with NCU00406, and they highlight the importance of this PAK-encoding gene to *Neurospora* biology. In *S. cerevisiae*, the VEL-related protein Cla4p is equally important to yeast biology, being required for normal bud morphology, pheromone responsiveness, filamentation, and invasive growth (10, 41, 89). In contrast, although *S. cerevisiae* Skm1p is necessary for normal resistance to hyperosmotic and other stresses (45, 98), this protein has not been implicated in the wide array of functions ascribed to Cla4p.

DISCUSSION

We identified 107 genes with the S/T protein kinase domain in the *Neurospora* genome. This number is similar to that for the yeast genome (~130), but smaller than that in humans (~380) and *Drosophila melanogaster* (~185) (58). Our comparative sequence analysis demonstrates that most *Neurospora* S/T kinases are well-conserved among organisms such as yeasts, animals, and plants. Our classification of S/T protein kinases is largely in agreement with a previous assessment of eukaryotic S/T kinases that included *N. crassa* (61). Six kinases showed no or low homology with those in yeast, animals, and plants, indicating that they may be specific to filamentous fungi. All of these were dispensable for the three major developmental processes analyzed (vegetative growth and asexual and sexual development), and none displayed chemical sensitivities, suggesting that these kinases may be involved in specific functions for optimized growth and development.

Our phenotypic analysis of kinase mutants provides important information about functions of S/T protein kinases during *Neurospora* growth and differentiation. The analysis was performed on only 86 kinase genes, because in three cases (NCU06419, NCU11235, and NCU11410), annotation errors resulted in removal of additional genes along with the kinase, and in 18 cases (*cdc28*, *div-18*, *hat-2*, *mus-9*, *mus-21*, *prk-11*, *rgb-40*, *stk-18*, *stk-24*, *stk-25*, *stk-27*, *stk-28*, *stk-33*, *stk-48*, *stk-52*, *stk-55*, *stk-58*, and *tkl-1*) the annotation was made after the start of this study. A total of 42/86 (49%) *Neurospora* kinase mutants did not exhibit any obvious phenotype during hyphal growth or asexual or sexual development, compared to 59/103 (57%) of transcription factor mutants studied in identical assays (13). In contrast, nine kinase genes (*cdc5*, *ck-1a*, *ck-1b*,

mdk-1, *pkc*, *prk-4*, *stk-1*, *stk-13*, and *stk-50*) were observed to be essential for fungal survival. In nearly all cases, these essential kinases were also found to be essential in *S. cerevisiae*, suggesting that these kinases are universal regulators of cell growth in fungi. The number of apparent essential S/T kinase genes (9/86) is approximately double that observed previously for transcription factor genes (4/103) (13), suggesting less redundancy in the S/T kinase gene class in *Neurospora*.

Our results revealed important differences between S/T kinases and transcription factors in the regulation of growth and development. A greater number of kinase mutants (44/77 viable mutants) were defective in at least one phenotype compared to transcription factor mutants (40/99 viable mutants). The difference is even greater when the number of genes involved in two or more growth/developmental functions is considered. Significantly more kinase genes (40%; 31/77 viable mutants) are involved in the regulation of two or more functions, compared to transcription factors (18%; 18/99 viable mutants). More than twice as many kinases as transcription factors (20 versus 9) are indispensable for all three functions. Thus, the data demonstrate that the impacts of kinases on fungal growth and differentiation are more dramatic than that on transcription factors, again, likely due to less functional redundancy in the kinases (7).

It is notable that 43% of the viable S/T kinases have no obvious roles during vegetative growth or asexual or sexual development. However, our chemical screening analysis revealed phenotypes for a total of 25 mutants, with 8 that did not exhibit defects in the growth and developmental assays. Of these eight, only one had been previously characterized, *gsk-3* (20). That earlier study linked *gsk-3* to glycogen metabolism and mitosis in *Neurospora*, but most of the phenotypes were either not analyzed in our study (arthro and microconidiation) or were relatively subtle. However, we did note altered resistance of the Δ*gsk-3* mutant to the microtubule-destabilizing chemical benomyl, perhaps related to its role in mitotic regulation. The remaining seven genes included CAMK1 kinase *camk-4*, CAMKL kinase *prk-10*, CDK kinase *prk-3*, CLK/SRPK/DYRK kinase *stk-54*, HAL family kinases *stk-5* and *stk-30*, and WEE kinase *stk-29*. The discovery of chemical phenotypes for these morphologically normal mutants should inform future functional studies of these genes. Our observation of novel phenotypes using chemical screens is consistent with the results of a large-scale chemical genomic screen using *S. cerevisiae*. In that study, genes having no obvious phenotypic consequences when deleted were essential for optimal growth under various chemical and environmental stress conditions (31).

Of all the mutants analyzed, Δ*apg-1* possessed the greatest number of chemical phenotypes (sensitivity to the four osmotic/oxidative stresses and FK-506), as well as defects in asexual and sexual growth and development. It has been demonstrated that the homolog of this kinase in *S. cerevisiae*, *ATG1*, is required for normal growth, sexual sporulation, autophagy, and accumulation of reactive oxygen species (85, 101), but *apg-1* had not been previously studied in *Neurospora*. Our results underscore the importance of this kinase to *Neurospora* environmental stress resistance, growth, and development. More work is needed to determine whether these functions are linked to the regulation of autophagy in this filamentous fungus.

Our study uncovered several S/T kinases that regulate different stages of sexual differentiation. A majority of the mutants that did not produce protoperithecia also possessed defects in hyphal growth and asexual development, indicating that abolishment of protoperithecial formation may often be linked to poor growth or lack of conidial differentiation. However, *cdc15* seems to be a unique kinase that is required only for protoperithecial formation. Our analysis also demonstrated that *stk-19* is primarily involved in the process by which the female trichogyne recognizes and fuses with the male cell. Since a G protein-coupled receptor, PRE-1, and the heterotrimeric Gα, Gβ, and Gγ proteins, GNA-1, GNB-1, and GNG-1, have been shown to regulate trichogyne recognition and fusion with male cells in previous studies (46, 51), it would be interesting to further investigate a possible interaction between STK-19 and PRE-1, GNA-1, GNB-1, or GNG-1.

We observed functional conservation for a number of S/T kinases when comparing *Neurospora* to other filamentous fungi. For example, PAKs and GCKs are known to regulate polarized hyphal growth in many filamentous and dimorphic fungi (for a review, see reference 8), and our analysis showed that three *Neurospora* PAKs/GCKs (*vel*, *stk-4*, and *prk-9*) are required for normal hyphal growth. In particular, the Δ*vel* PAK mutant had a colony morphology (reduced growth and shortened aerial hyphae) that has also been observed in corresponding mutants in the filamentous fungi *Magnaporthe grisea* and *Claviceps purpurea* (54, 77).

We noted examples of interesting variations in the functions of S/T kinases implicated in mitotic regulation in *Neurospora* and the filamentous fungus *Aspergillus nidulans*. S/T kinases homologous to Cdc2 are at the top of the phosphorylation cascade that regulates mitosis in numerous eukaryotes (reviewed in reference 65). Cdc2 phosphorylates S/T kinases in three different families: Aurora, Polo-like, and NEK. The *Neurospora CDC2* homolog (*cdc28*) was not annotated at the initiation of our study, but the *A. nidulans* homolog (*nimX*) is an essential gene (97). The downstream Aurora and the Polo-like kinase genes are essential in *Neurospora* (*stk-13* and *cdc5* [this study]), *A. nidulans* (*Aurora* and *plkA* [3, 28]) and *S. cerevisiae* (47, 62). However, although the NEK *nimA* is required for mitotic entry and is thus essential in *A. nidulans* (96), it is dispensable for viability in *Neurospora* (this study), as well as the yeasts *Schizosaccharomyces pombe* (50) and *S. cerevisiae* (43). The different requirement for the NEK in the two filamentous fungi is perhaps surprising, in light of the ability of the *Neurospora* gene to complement the *A. nidulans nimA* mutation (75). However, accumulating evidence suggests that the functions of NEKs are not restricted to mitotic entry, as first demonstrated in *A. nidulans*, but also influence other aspects of mitotic control, in roles that are shared with other kinases (65).

Another example of a difference between *Neurospora* and *A. nidulans* concerns the role of the STE11 family kinase *cdc15* (in *Neurospora*) and *sepH* (in *A. nidulans*). *sepH* regulates septum formation and hyphal differentiation in *A. nidulans* (9, 30). In contrast, mutation of *cdc15* did not lead to defects in growth of vegetative hyphae in *Neurospora*, but it blocked the formation of protoperithecia during the sexual cycle. Since *sepH* regulates septum formation through control of cytoskeletal structure in *A. nidulans*, it is possible that a role for *cdc15* in reorganizing the cytoskeleton is only observed during protoperithecia formation in *Neurospora*.

The observation that 71% of the S/T kinases mutated in our study were either essential or necessary for normal growth,

development, or chemical resistance underscores the central importance of S/T protein kinases to *Neurospora* biology. Further investigations will illuminate specific roles for individual kinases, reveal those that operate in shared pathways, and determine the extent of functional conservation between S/T kinases from *Neurospora* and other organisms.

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