Published in final edited form as:
Mol Microbiol. 2012 October ; 86(2): 284–302. doi:10.1111/j.1365-2958.2012.08193.x.

Functional control of the *Candida albicans* cell wall by catalytic protein kinase A subunit Tpk1

S Fanning[a], **W Xu**[a], **C Beaurepaire**[b], **JP Suhan**[a], **A Nantel**[b,c], and **AP Mitchell**[a,*]

[a]Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, USA

[b]Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, Canada

[c]Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada

SUMMARY

The cyclic AMP-protein kinase A pathway governs numerous biological features of the fungal pathogen *Candida albicans*. The catalytic protein kinase A subunits, Tpk1 (orf19.4892) and Tpk2 (orf19.2277), have divergent roles, and most studies indicate a more pronounced role for Tpk2. Here we dissect two Tpk1-responsive properties: adherence and cell wall integrity. Homozygous *tpk1/tpk1* mutants are hyperadherent, and a Tpk1 defect enables biofilm formation in the absence of Bcr1, a transcriptional regulator of biofilm adhesins. A quantitative gene expression-based assay reveals that *tpk1/tpk1* and *bcr1/bcr1* genotypes show mixed epistasis, as expected if Tpk1 and Bcr1 act mainly in distinct pathways. Overexpression of individual Tpk1-repressed genes indicates that cell surface proteins Als1, Als2, Als4, Csh1, and Csp37 contribute to Tpk1-regulated adherence. Tpk1 is also required for cell wall integrity, but has no role in the gene expression response to cell wall inhibition by caspofungin. Interestingly, increased expression of the adhesin gene *ALS2* confers a cell wall defect, as manifested in hypersensitivity to the cell wall inhibitor caspofungin and a shallow cell wall structure. Our findings indicate that Tpk1 governs *C. albicans* cell wall properties through repression of select cell surface protein genes.

Keywords

cyclic AMP; adherence; biofilm; cell wall integrity

INTRODUCTION

The cyclic AMP-protein kinase A pathway has a pivotal role in *C. albicans* morphogenesis and infection biology (Biswas *et al.*, 2007). The pathway responds to diverse environmental signals, including CO_2, bacterial peptidoglycan, and the quorum sensing molecule farnesol (Hogan & Muhlschlegel, 2011). Outputs are equally diverse, and include the yeast-to-hypha morphogenesis program, the white-opaque cell type switch, biofilm formation, binding and damage to epithelial and endothelial cells, and pathogenic potential in several infection models (D'Souza & Heitman, 2001, Leberer *et al.*, 2001, Bahn & Sundstrom, 2001, Cassola *et al.*, 2004, Cloutier *et al.*, 2003). Adenylyl cyclase is thus considered a central integrator of

*Corresponding author: Aaron Mitchell, 200B Mellon Institute, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213. apm1@cmu.edu. Telephone 001- 412-268-5844. Fax 412-268-7129.

NCBI GEO database: GSE38846
Complete microarray data can also be downloaded from http://www.cmu.edu/bio/faculty/mitchell.html.

key signals that ultimately governs the way that *C. albicans* interacts with its host and its competitors (Hogan & Muhlschlegel, 2011).

The intracellular responses to cyclic AMP pathway are mediated by two catalytic subunits of cyclic AMP-dependent protein kinase, Tpk1 and Tpk2 (Cloutier et al., 2003). These protein kinases are activated by the cyclic AMP-induced dissociation of the regulatory subunit, Bcy1 (Cassola *et al.*, 2004), a highly conserved mechanism (D'Souza & Heitman, 2001). Tpk1 and Tpk2 then phosphorylate targets to effect changes in cell biology and gene expression.

Tpk1 and Tpk2 have distinct functions for the most part, as assessed by comparison of *tpk1Δ/tpk1Δ* and *tpk2Δ/tpk2Δ* mutant phenotypes. Tpk2 is required for hyphal development in liquid (Bockmuhl et al., 2001) while Tpk1 is required only for hyphal formation on solid media (Bockmuhl et al., 2001). Tpk2 is required for agar invasion; Tpk1 is not (Bockmuhl et al., 2001). Tpk2 governs the qualitative nature of filamentous cells (pseudohyphal to hyphal cell ratio), and thus impacts biofilm formation; Tpk1 does not (Giacometti *et al.*, 2011). The two catalytic subunits also have contrasting roles in glycogen storage as well as resistance to saline, heat, and oxidative stresses (Giacometti et al., 2009). In general, Tpk2 has a more prominent role than Tpk1 in *C. albicans* biology, most likely because it is the more abundant isoform (Cloutier et al., 2003, Souto *et al.*, 2006, Giacometti et al., 2011).

Our interest is in the *C. albicans* cell surface and how it contributes to biofilm formation and drug sensitivity. In that context, we find Tpk1 noteworthy, because it is required for normal levels of both resistance to the cell wall inhibitor caspofungin (Blankenship *et al.*, 2010) and, as we show here, adherence to a silicone substrate. Tpk2 does not have measurable impact on either process. These observations prompted us to investigate the mechanistic basis for these roles of Tpk1. Our findings reinforce the idea that Tpk1 has a selective role in *C. albicans* biology. Most importantly, our results connect defined Tpk1 transcriptional targets to specific biological features, thus strengthening our functional understanding of a central regulatory pathway's outputs and their relevance to infection biology.

RESULTS

Control of C. albicans silicone adherence by protein kinase genes

In order to define the genetic control of adherence to silicone, we assayed a panel of 70 insertion mutants in protein kinase-related (PK) genes (Blankenship et al., 2010) for altered adherence. A silicone substrate was used to represent the surface of implanted medical devices, such as a venous catheter. Mutants that were hyperfilamentous, aggregated, or grew poorly were not assayed (7 in total). We found that 22 PK mutants had significantly decreased cell-surface adherence, 5 had increased adherence, and 36 showed no significant difference from the wild type under our assay conditions (Figure 1A). Hence, a large fraction of PK mutants have phenotypic impact in this assay. These results are consistent with previous studies in which a high frequency of pronounced phenotypes were found among PK mutants of *C. albicans* (Blankenship et al., 2010), *S. pombe* (Bimbo *et al.*, 2005) and *N. crassa* (Park *et al.*, 2011).

A previous study indicated that a large fraction of PK mutants had defects in cell wall integrity, as evidenced by hypersensitivity to caspofungin (Blankenship et al., 2010). We considered the possibility that cell wall defects and altered adherence may be linked. For example, cell wall perturbation induces *ALS1* expression (Blankenship et al., 2010, Bruno *et al.*, 2006, Gregori *et al.*, 2011), and Als1 promotes adherence to silicone (Finkel et al., 2012). In fact, over 29% of the PK mutants with altered adherence are also hypersensitive to

cell wall perturbation, including 4 out of 5 of the PK mutants with increased adherence. This finding suggests that altered adherence and cell wall defects may be functionally related.

Elevated adherence has seldom been investigated in *C. albicans*, and we reasoned that this phenotype may provide unique insight into cell wall regulatory mechanisms. We focused in particular on the *tpk1::Tn/tpk1::Tn* insertion mutant, because it displayed the most pronounced increase in adherence (Figure 1A). The *tpk1::Tn/tpk1::Tn* insertion mutant phenotype differed from that of the *tpk2::Tn/tpk2::Tn* insertion mutant (Figure 1A), thus suggesting that this phenotype is one more reflection of the divergent functions of Tpk1 and Tpk2. We verified the hyperadherent phenotype with assays of a derivative of the original "Ura-blaster" *tpk1Δ/tpk1Δ* mutant (strain HPY300U), with multiple independent *tpk1Δ/tpk1Δ* deletion mutants in either the SC5314 or BWP17 strain backgrounds, and through complementation tests (Figure 1B and data not shown). Therefore, Tpk1 is a negative regulator of silicone adherence.

Tpk1 function in biofilm formation

Surface adherence can lead to biofilm formation. Therefore we sought to determine whether increased adherence of a *tpk1Δ/tpk1Δ* mutant might affect biofilm formation. Wild-type strains form biofilms efficiently under our assay conditions; few planktonic cells are detectable in biofilm supernatants (Nobile *et al.*, 2006b). Thus an increase in adherence over the wild type would not be detectable as an improvement in biofilm formation. In addition, we observed no unusual structural features of *tpk1Δ/tpk1Δ* mutant biofilms (data not shown). Therefore we turned to a *bcr1Δ/bcr1Δ* mutant background, which is defective in adherence and biofilm formation. Bcr1 is a transcription factor that is required for expression of numerous cell surface protein genes (Finkel et al., 2012). We reasoned that, if a *tpk1Δ/tpk1Δ* defect led to increased adherence in a *bcr1Δ/bcr1Δ* mutant background, then an improvement in biofilm formation might be detectable.

To test this prediction, we compared biofilm formation among a wild-type reference strain, a *bcr1Δ/bcr1Δ* mutant, a *bcr1Δ/bcr1Δ tpk1Δ/tpk1Δ* double mutant, and a *bcr1Δ/bcr1Δ tpk1Δ/tpk1Δ+TPK1* complemented control strain. The ability to form a biofilm was severely impaired in the *bcr1Δ/bcr1Δ* strain, as visualized by scanning electron microscopy (SEM), but was restored in *bcr1Δ/bcr1Δ tpk1Δ/tpk1Δ* double mutant (Figure 2A, B). The complemented *bcr1Δ/bcr1Δ tpk1Δ/tpk1Δ+TPK1* strain behaved similarly to the *bcr1Δ/bcr1Δ* mutant, and thus confirmed that the *tpk1Δ/tpk1Δ* mutation was responsible for improved biofilm formation (Figure 2A, B). These findings suggest the hypothesis that the increased adherence resulting from a defect in *TPK1* can improve biofilm formation.

The analysis of double mutants is in essence an epistasis test, and the results above show that a *tpk1Δ/tpk1Δ* defect is epistatic to a *bcr1Δ/bcr1Δ* defect for the biofilm formation phenotype. Epistasis is often interpreted in the context of gene regulation (Roth *et al.*, 2009). Therefore, we also examined epistasis at the level of gene expression. Specifically, we measured expression of a panel of 117 genes through nanoString analysis of RNA levels (Geiss *et al.*, 2008). Our gene set included several Bcr1- and stress-responsive genes as well as the control gene *TDH3* for normalization purposes (Table S1). Cells were grown in our biofilm medium (Spider medium) at 37°C; conditions used previously for microarray and nanoString analysis of the *bcr1Δ/bcr1Δ* strain (Nobile & Mitchell, 2005, Finkel et al., 2012). We compared gene expression levels among four strains: the wild type, a *tpk1Δ/tpk1Δ* mutant, a *bcr1/bcr1Δ* mutant, and a *bcr11Δ/bcr1Δ tpk1Δ/tpk1Δ* double mutant. We used the combined expression levels of all genes in the set as a basis for determining epistasis. Comparison of gene expression in the *tpk1Δ/tpk1Δ* and *bcr1Δ/bcr1Δ* strains (Figure 3A) shows many substantial differences in expression levels. Agreement or divergence between these two expression datasets can be represented by the correlation coefficient (R^2) value of

0.53. When we compared the *tpk1Δ/tpk1Δ bcr1Δ/bcr1Δ* double mutant to the *tpk1Δ/tpk1Δ* mutant, we obtained an R^2 value of 0.67. The R^2 value of 0.67 is greater than 0.53; a simple interpretation is that the *tpk1Δ/tpk1Δ* phenotype is partially manifested in the double mutant, and thus that the *tpk1Δ/tpk1Δ* defect is partially epistatic to the *bcr1Δ/bcr1Δ* defect. When we compared the *tpk1Δ/tpk1Δ bcr1Δ/bcr1Δ* double mutant to the *bcr1Δ/bcr1Δ* mutant, we obtained an R^2 value of 0.83 (Figure 3C). Following our previous logic, we infer that the *bcr1Δ/bcr1Δ* phenotype is partially manifested in the double mutant, and thus that the *bcr1Δ/bcr1Δ* defect is partially epistatic to the *tpk1Δ/tpk1Δ* defect. Comparison of the double mutant to the wild-type strain indicates that the two mutations to not precisely counterbalance each other (Figure 3D; R^2 of 0.67). Therefore, each mutation is partially epistatic to the other at the level of gene expression. This finding is not consistent with the idea that Tpk1 and Bcr1 act at successive steps of a single pathway. Instead, it is consistent with a model in which Tpk1 and Bcr1 act, for the most part, independently of one another.

Identification of TPK- regulated genes

Our findings above suggest that Tpk1 affects a different pathway from Bcr1. Therefore, we sought to define Tpk1 pathway target genes on a genome-wide scale. We conducted a microarray comparison between the *tpk1::Tn/tpk1::Tn* insertion mutant and the *tpk1::Tn/tpk1::Tn+TPK1* complemented strain (NCBI GEO database: GSE38846). Fourteen of the targets identified by microarray were verified by RT-PCR (Figure 4A). Interestingly, 8 out of the 14 genes that were most up-regulated in the *tpk1::Tn/tpk1::Tn* mutant specify known or predicted cell wall or cell surface localized proteins, including adhesins Als1, Als2, and Als4. We verified that *ALS1*, *ALS2*, and *ALS4* RNA levels were up-regulated in the Ura-blaster *tpk1Δ/tpk1Δ* isolate (data not shown). Because our interest is in phenotypes related to the cell wall and cell surface, we focused on the up-regulated genes for functional analysis.

Relationship of Tpk1-repressed genes to adherence

We considered the specific hypothesis that Tpk1 defects cause increased adherence through up-regulation of genes that promote adherence. To test this hypothesis, we created derivatives of the adherence-defective *bcr1Δ/bcr1Δ* mutant that overexpressed individual Tpk1-repressed genes and assayed each strain for adherence and biofilm formation. We observed that overexpression of *ALS1*, *CSH1*, *CSP37*, *ALS2*, or *ALS4* caused a significant increase in adherence in the *bcr1Δ/bcr1Δ* background (Figure 4B). In addition, overexpression of each of the five genes in the *bcr1Δ/bcr1Δ* mutant improved biofilm formation substantially, as determined by SEM visualization (Figure 5) and, in most cases, biofilm biomass measurements (Figure 4C). *CSH1* was the one anomalous case: biofilms of the *CSH1*-overexpressing *bcr1Δ/bcr1Δ* mutant fragmented under typical handling conditions, so an increase in biomass could not be documented (Figure 4C). Although some of the strains that overexpressed other Tpk1-repressed genes seemed to trend toward increased adherence or biofilm formation, these increases were not statistically significant. We conclude that five Tpk1-repressed genes, *ALS1*, *CSH1*, *CSP37*, *ALS2*, and *ALS4*, promote substrate adherence and biofilm formation.

Relationship of Tpk1-repressed genes to cell wall integrity

We reported previously that a *tpk1::Tn/tpk1::Tn* insertion mutant is hypersensitive to caspofungin (Blankenship et al., 2010). Our isolate of the original Ura-blaster *tpk1Δ/tpk1Δ* mutant did not share this phenotype. To determine whether Tpk1 is required for caspofungin tolerance, we examined multiple independent insertion and deletion mutants in both the BWP17 and SC5314 backgrounds. All were caspofungin hypersensitive, and the phenotype was rescued by complementation (Figure 6A and (Blankenship et al., 2010)). The complementation results indicate that Tpk1 defects are the cause of the phenotype. We first

considered the possibility that Tpk1 may be required for cell wall integrity gene expression. Microarray analysis indicated that all of the genes that were most highly up- or down-regulated in response to caspofungin in the complemented strain responded similarly in the *tpk1::Tn/tpk1::Tn* mutant (Figure 7 and NCBI GEO database: GSE38846). In a few cases in which the mutant did not show a significant change in expression of a gene (white bars in Figure 7), the trends and mean expression changes were still similar to that of the complemented strain. Therefore, Tpk1 is not required for cell wall integrity gene expression.

We considered a second explanation for the *tpk1/tpk1* mutant caspofungin hypersensitivity: altered expression of cell wall and cell surface proteins in *tpk1/tpk1* mutants may perturb cell wall structure and cause caspofungin hypersensitivity. To test this hypothesis, we created a panel of strains that overexpressed the top Tpk1-repressed genes in an otherwise wild type background, and assayed their sensitivity to caspofungin. Strikingly, overexpression of *ALS2* specifically conferred substantial caspofungin hypersensitivity (Figure 6B). We observed the same phenotype among several *ALS2* overexpressing transformants (data not shown). In addition, among the *bcr1Δ/bcr1Δ* strains that overexpress Tpk1-repressed genes, we observed caspofungin hypersensitivity specifically when *ALS2* was overexpressed (data not shown). These findings suggest that one role of Tpk1 in cell wall integrity is to prevent *ALS2* expression.

It seemed possible that the cell wall perturbation conferred by a Tpk1 defect might be evident by electron microscopy. Indeed, this analysis revealed that the *tpk1Δ/tpk1Δ* mutant has a cell wall that is half the depth of the wild-type cell wall (Figure 8A, B). This mutant phenotype was complemented by introduction of a wild-type *TPK1* allele (Figure 8C). The shallow cell wall phenotype was also manifested by the strain that overexpressed *ALS2* (Figure 8D), but not the related gene *ALS4* (Figure 8F). Consistent with the findings of Hoyer and colleagues (Zhao *et al.*, 2005), we were unable to construct a homozygous deletion mutant of *ALS2*. Deletion of one allele of *ALS2* resulted in a heterozygous *ALS2* strain with a cell wall depth significantly greater than the wild-type strain (Figure 8E). Overexpression of the other Tpk1-repressed *ALS* gene, *ALS1*, caused cell wall depth that was intermediate between that of the *tpk1Δ/Δ* mutant and the wild type (Figure S1).

These results indicate that Tpk1 promotes both cell wall integrity and cell wall depth, and that Tpk1-dependent repression of *ALS2* is one mechanism that mediates both functions.

DISCUSSION

Cell-substrate adherence is pivotal for biofilm formation, a major source of *C. albicans* infection (Finkel & Mitchell, 2011). We have described here a quantitative characterization of protein kinase mutants that identified many new regulators of adherence. Our findings define a function for many protein kinase genes that have not been extensively characterized and, in addition, connect adherence for the first time to several known signalling and cell biological pathways. We have focused in particular on Tpk1, and discern a novel connection between adherence, cell wall integrity, and cell wall structure. Several Tpk1-responsive genes contribute to adherence, and one Tpk1-responsive gene, the adhesin gene *ALS2*, has significant roles in adherence as well as cell wall integrity and structure (Figure 9).

Previous analysis of *tpk1/tpk1* mutants has implicated Tpk1 in diverse metabolic and cellular processes (Souto et al., 2006, Giacometti et al., 2009, Giacometti et al., 2011, Bockmuhl et al., 2001, Cloutier et al., 2003, Cassola et al., 2004, Park et al., 2005). Stress sensitivity is one general theme, and our microarray data provide some functional insight. For example, the *tpk1/tpk1* glycogen deficiency (Giacometti et al., 2009) may arise from increased expression of *GBD1*, which specifies a glycogen debranching enzyme that promotes

glycogen degradation (Teste *et al.*, 2000). The *tpk1/tpk1* salt sensitivity (Giacometti et al., 2009) may arise from increased expression of *GAC1*, which specifies a protein phosphatase regulatory subunit whose *S. cerevisiae* ortholog, when overexpressed, confers salt sensitivity (Wu *et al.*, 2001). We cannot point to a cause of the *tpk1/tpk1* peroxide sensitivity (Giacometti et al., 2009), but elevated expression of oxidative stress genes *HSP12*, *GPX2*, and *orf19.251* may result from elevated endogenous reactive oxygen species levels. Cell-surface interaction is another recurring theme among Tpk1-responsive properties. Tpk1 was known to promote formation of hyphae on solid media (Bockmuhl et al., 2001), not in liquid, and our studies here show that Tpk1 governs surface adherence as well. Interestingly, our microarray data provide convergent evidence for a close Tpk1-cell wall connection, which is related logically to cell-surface interaction. Among 33 significantly up-regulated genes in the *tpk1::Tn/tpk1::Tn* mutant, there was pronounced enrichment for cell wall-related functions (GO term "fungal-type cell wall," 8 genes, P-value 4.29×10^{-6}). Thus Tpk1 is tied to cell surface properties through both cellular phenotypes and gene expression targets.

Tpk1 is well established as one of the two cyclic AMP-dependent protein kinase catalytic subunits in *C. albicans* (Cloutier et al., 2003), yet we have found no alteration of adherence or biofilm formation in mutants of the upstream genes *CDC25* (orf19.6926) and *CDC35/CYR1* (orf19.5148) (unpublished results). We have also used gene expression changes as a measure of phenotype. Among the 46 genes that are up-regulated at least 2-fold in the *tpk1::Tn/tpk1::Tn* mutant, we have microarray data from a *cdc35Δ/cdc35Δ* for 23 genes (Harcus *et al.*, 2004). Among those 23 genes, almost all are up-regulated in the *cdc35Δ/cdc35Δ* mutant compared to the wild type (Figure S2), including *CSP37*, *ECM331*, and *IHD1*. This correlation between Tpk1-repressed genes and Cdc35-repressed genes is consistent with the current understanding of the cyclic AMP pathway. Given that Tpk2 can compensate for the loss of Tpk1 (Bockmuhl et al., 2001), it seems reasonable that some phenotypes of *tpk1/tpk1* mutants may be distinct from those of *cdc35/cdc35* mutants.

We have used a *bcr1Δ/bcr1Δ* mutant as an analytical tool in our studies because it has reduced adherence and biofilm formation capability. Nonetheless, because Bcr1 and Tpk1 govern related phenotypes, it may seem possible that they act in the same pathway. Our data provide no support for that idea. Specifically, our nanoString data show little correlation between the genes up- and down-regulated in the two mutants. One of the few prominent exceptions is *ALS1*, which is down-regulated in the *bcr1Δ/bcr1Δ* mutant and up-regulated in the *tpk1Δ/tpk1Δ* mutant (see Table S1). If we examine *ALS1* expression specifically, the *bcr1Δ/bcr1Δ* phenotype is epistatic to the *tpk1Δ/tpk1Δ* phenotype, in keeping with the idea that Bcr1 functions downstream of Tpk1. That conclusion is consistent with the genome-wide chromatin immunoprecipitation data from Nobile and colleagues (Nobile *et al.*, 2012), which indicates that Bcr1 binds directly to the *ALS1* promoter. However, those data also indicate that four additional transcription factors bind to the *ALS1* promoter, including the known cyclic AMP pathway target Efg1 (Sonneborn *et al.*, 2000, Bockmuhl & Ernst, 2001, Stoldt *et al.*, 1997). Thus the *ALS1* promoter may be a region at which multiple signalling pathways interact, and thus a challenging choice for deductions about pathway relationships. Our overall inference, based on non-correlation of Tpk1- and Bcr1-responsive genes, is that these two gene products largely act independently to govern gene expression.

Analysis of the Tpk1-repressed genes in the chromatin immunoprecipitation dataset from Nobile and colleagues (Nobile *et al.*, 2012) has the potential to define the transcription factors that mediate regulation by Tpk1. Among the 14 Tpk1-repressed genes in Figure 4A, six genes had no detectable binding of any transcription factor studied, two genes were bound only by Ndt80, one gene was bound only by Tec1, one gene was bound by both Ndt80 and Tec1, and four genes were bound by Ndt80, Efg1, Bcr1, and some combination

of Brg1 and Tec1. Thus there is no unequivocal candidate for a mediator of Tpk1 responsiveness, but Ndt80 binds to more of these genes than any other transcription factor examined (Nobile *et al.*, 2012). A simple model is that Ndt80 protein levels or activity increases in the absence of Tpk1, and activates a large subset of Tpk1-repressed genes.

Functional analysis indicates that several Tpk1-repressed gene products contribute to adherence. Among them, Als1, Als2, and Als4 are canonical adhesins, in that they are large, GPI-linked cell surface proteins. All three, like other Als family members, have been shown to mediate adherence to several substrates, using both overexpression approaches and underexpression or null mutant approaches (Finkel et al., 2012, Nobile *et al.*, 2006a, Kamai *et al.*, 2002, Zhao *et al.*, 2004, Green *et al.*, 2004, Spellberg *et al.*, 2005, Nobile *et al.*, 2008a, Dwivedi *et al.*, 2011). The other relevant targets, Csh1 and Csp37, do not have structural features of known adhesins, such as a signal sequence or GPI anchor addition signal. However, both have been found associated with the cell wall (Urban *et al.*, 2003, Pitarch *et al.*, 2002, Singleton *et al.*, 2001), so their localization is consistent with a direct role in adherence. In addition, deletion mutants of either gene display reduced adherence (Singleton et al., 2001, Sentandreu *et al.*, 1997). Finally, both genes are down-regulated in several recently characterized biofilm-defective mutants (Nobile et al., 2012). Thus our conclusions regarding Tpk1-repressed genes that govern adherence, based on overexpression assays in a *bcr1Δ/bcr1Δ* mutant, are consistent with functional assignments from independent studies and null mutant analysis. The mechanistic connection between Tpk1 and adherence fits a well-accepted paradigm: inactivation of Tpk1 causes elevated expression of several surface proteins that may mediate surface interaction through direct binding or, perhaps, through more global effects on cell wall structure.

Tpk1-mediated cell wall integrity has two surprising features. First, Tpk1 seems to have no role in the cell wall integrity gene expression response: the mutant and complemented strains both induced and repressed the same genes after caspofungin treatment. Second, our studies point toward a single cell surface protein, Als2, whose increased expression mediates caspofungin hypersensitivity and the correlated phenotype, a shallow cell wall. A heterozygous deletion of *ALS2* yielded a cell wall depth thicker than the wild type. A correlation between cell wall depth and integrity was first established by Plaine et al. (Plaine *et al.*, 2008) in a survey of cell wall protein gene mutants. The intriguing feature of the cell wall aberration in the *tpk1/tpk1* mutant is that it apparently does not trigger a cell wall integrity gene expression response. Many other caspofungin-hypersensitive regulatory mutants have a partially activated response (Blankenship et al., 2010), as expected if their cell wall biosynthetic defect generates the same regulatory signal as addition of an exogenous cell wall inhibitor. Apparently one kind of chronic cell wall defect remains undetected by the *C. albicans* cell, as assayed by levels of gene expression.

Our observations point toward a novel function for Als2 in cell wall structure. There is extensive similarity between Als2 and Als4 proteins as well as their respective genetic loci (Zhao *et al.*, 2005, Hoyer *et al.*, 2008). However, only one of the two *ALS2* alleles could be deleted by Zhao et al. (Zhao et al., 2005), and they proposed that Als2 may be essential for viability. Our findings, based on *ALS2* overexpression, suggest specifically that the essential role of Als2 may be in cell wall biogenesis. One might argue that overexpression of any cell surface protein could cause such cell wall aberrations for nonspecific reasons, unrelated to natural protein function. However, Als4 is ~60% identical to Als2, yet its overexpression does not affect either caspofungin sensitivity or cell wall depth. One might suppose that *TDH3-ALS2* is overexpressed to a much greater extent than *TDH3-ALS1* or *TDH3-ALS4*, but in fact the opposite is true: *TDH3-ALS2* increased *ALS2* RNA levels 4-fold, whereas *TDH3-ALS1* and *TDH3-ALS4* increased *ALS1* and *ALS4* RNA levels 77- and 97-fold, respectively

(unpublished results). Thus our results argue that effects of *ALS2* overexpression are specific, and connect Als2 to a process that is essential for viability.

Most studies of Tpk1 and Tpk2 have revealed many prominent roles for Tpk2 yet few for Tpk1 (Souto et al., 2006, Giacometti et al., 2009, Giacometti et al., 2011, Bockmuhl et al., 2001, Cloutier et al., 2003, Cassola et al., 2004, Park et al., 2005). Our findings help to understand this distinction. First, our microarray data reveal that a fairly small set of genes responds to Tpk1 deficiency in growing cells. In fact, a Tpk1 deficiency has little gene expression impact on the perturbations caused by a Bcr1 defect or addition of a cell wall inhibitor. Second, we show that Tpk1 deficiency has profound impact on adherence, but causes a phenotype that is not easily detected as a defect. Rather, the hyperadherence is most readily detected as a restoration of biofilm formation ability. Together, our findings suggest that the cell wall is a major target of Tpk1 activity, and that much of this role is mediated by a few selective Tpk1-responsive gene expression changes.

EXPERIMENTAL PROCEDURES

Media and Strain Construction

C. albicans strains were grown on yeast extract-peptone dextrose (YPD) (2% Bacto Peptone, 2% dextrose,1% yeast extract and supplemented with 80μg/ml uridine for Ura$^-$ strains) or on defined synthetic dextrose medium (2% dextrose, 6.7% YNB with ammonium sulfate, and auxotrophic supplements). For transformation procedures cells were selected on SC medium minus the appropriate amino acid(s). caspofungin (Merck) was added to media at 125ng/ml.

All *C. albicans* strains used in this study are listed in Table 1 and Table S2 and primer sequences are listed in Table 2. Protein kinase insertion mutant strains utilized were generated in (Blankenship et al., 2010) and are listed in Table S2. Unless otherwise indicated, newly constructed strains were derived from BWP17 (Wilson *et al.*, 1999). Strain SF1130, a prototrophic deletion (*tpk1Δ/tpk1Δ*), was constructed by PCR-directed gene deletion (Wilson et al., 1999) employing long oligonucleotides TPK1-5DR and TPK1-3DR. A double deletion of *BCR1* and *TPK1*, SF1151, was constructed by deleting both *TPK1* alleles using PCR directed gene deletion in an auxotrophic bcr*1Δ/bcr1Δ* (SF803) strain.

To complement deletions, PCR fragments of the gene to be complemented, including 1000bp upstream of the start codon and approximately 700bp downstream of the stop codon, were amplified from BWP17 genomic DNA. Complementation primers were 60–70bp in length and comprised a 20–30bp gene specific sequence and a 40mer sequence (upstream primer-TTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCT, downstream primer-*TCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAG)* to direct in vivo recombination into plasmid pDDB78 (Ma *et al.*, 1987). The complementation PCR product was co-transformed into the *S. cerevisiae* BY4741Δtrp strain with an EcoRI/NotI restricted pDDB78 plasmid. The resulting complementing clone was amplified in *E. coli*, digested with NruI to direct insertion of the complement to the HIS1 locus and transformed into the deletion strains. Presence of wild-type was detected by colony PCR. NruI-digested pDDB78 was also transformed into the deletion strains to generate prototrophic marker-matched strains for comparison with complemented strains.

The SAT1 flipper (Reuss *et al.*, 2004) was used to generate a *TPK1* gene deletion in the clinical isolate SC5314, SF1358. The *TPK1* deletion cassette was constructed by insertion of a KpnI-XhoI fragment (approx 500bp in length of the region upstream of *TPK1*) and a SacII-SacI fragment (approximately 500bp in length of the region downstream of *TPK1*) into pSFS-2A. The resulting plasmid was linearized with KpnI and SacI and transformed

into *C. albicans* SC5314 and plated on YPD containing 200μg/ml clonNAT and screened by PCR. Positive transformants were grown overnight in YPD containing maltose as the sole carbon source to induce excision of the cassette and plated on YPD. Colonies that could no longer grow on 100μg/ml NAT were identified as having excised the cassette and were transformed again with linearized plasmid to obtain homozygous deletions.

To construct over-expressing strains, the *NAT1-TDH3* promoter plasmid pCJN542 (Nobile *et al.*, 2008b) was employed. Primers were designed to have 100bp of homology to 500bp upstream into the promoter region and 100bp of homology from the start codon of the gene to be overexpressed. In cases where there was a gene upstream (within the 500bp upstream) a smaller fragment was generated to ensure other orfs were not compromised. PCR products were generated using pCJN542 as the template. Homology allowed for homologous recombination of the cassette directly upstream of the gene to be overexpressed with the *TDH3* promoter driving expression in place of the genes natural promoter. Transformants were plated on YPD+400μg/ml clonNAT plates and transformants were PCR checked using a gene-specific forward detection primer and a reverse primer specific to the *NAT* gene. Genotypes of all strains constructed were verified by PCR and many were also verified by RT-PCR. In all cases, marker-matched strains were generated and most appropriate wild-type strains were used as reference strains (e.g. DAY185 for prototrophic mutant strains) for phenotype assays.

RNA Isolation

Overnight cultures of cells were diluted to an OD_{600nm} of 0.2 in 100 ml fresh YPD medium (or other appropriate medium for the experiment). Cultures were grown at 30°C with shaking to an OD_{600nm} of 1 or at 37°C for 8 hours. For cell wall integrity studies the 100ml cultures were the split into two cultures. 125ng/ml of caspofungin was added. To the other an equal volume of water was added and the cultures were incubated for 30 minutes. Cells were harvested by vacuum filtration and flash frozen in a dry ice/EtOH bath. Cells were frozen on filters at −80°C.

RNA was extracted using the RNeasy Kit (Qiagen Cat.No. 74104) following the manufacturer's protocol for Purification of Total RNA from Yeast with the following modifications. Cells were resuspended from filters with 1.5 ml ice-cold dH_2O followed by 10–15 seconds of vigorous vortexing. Resuspended cells were transferred to a 1.5 ml tube and spun down according to the manufacturer's protocol. For cell disruption, the manufacturer's protocol for mechanical disruption was used and cells were beaten with a Next Advance Bullet Blender for 3 min at 4°C to maximize cell lysis.

Microarray

Microarray expression analysis was performed as previously described (Nantel *et al.*, 2006). Individual hybridization experiments were performed from multiple independent RNA samples of JJH384 and JJH233 (*tpk1::Tn/tpk1::Tn* and *tpk1::Tn/tpk1::Tn +TPK1*) with and without caspofungin treatment. Microarray data was verified by RT-PCR of a number of the top targets found by microarray. The microarray data has been deposited in the NCBI GEO database under accession number GSE38846.

Quantitative RT PCR

10 μg total RNA was treated with the DNA-free kit (Ambion) followed by first-strand cDNA synthesis from half of the DNA-free RNA using the AffinityScript multiple temperature cDNA synthesis kit (Stratagene). Absence of DNA contamination was confirmed using control sets for which reverse transcriptase was omitted from the cDNA reaction.

Primer3 software (http://frodo.wi.mit.edu/) was used to design primers for all genes including *TDH3* which was used as a reference gene for normalisation. Primers to distinguish between the *ALS* genes were as per (Green *et al.*, 2004) (Table 2). 2X iQ SYBR Green Supermix (Bio-Rad), 1 μl of first-strand cDNA reaction mixture, and 0.1 μM of primers were mixed in a total volume of 50μl per reaction. Real-time PCR was performed in triplicate using an iCycler iQ real-time PCR detection system (Bio-Rad). The program for amplification had an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 45 s and 58°C for 30 s. Product amplification was detected using SYBR Green fluorescence during the 58°C step, and specificity of the reaction was monitored by melt-curve analysis following the real-time program. Gene expression was determined using Bio-Rad iQ5 software (ΔΔ*CT* method).

Cell-Surface Adherence

The method outlined by (Finkel et al., 2012) was followed to assess cell-surface adherence for a library of protein kinase insertion mutants constructed in (Blankenship et al., 2010). Briefly, the Fluxion Bioflux (TM) 200 (Fluxion Biosciences, San Francisco, CA), a flow device consisting of microfluidic channels permitting steady flow through a flow chamber comprised of a glass coverslip fused to a fluidic channel constructed from polydimethylsiloxane, was employed to analyse cell to surface adherence of the strains. Overnight cultures of wild-type and mutant strains were diluted to an OD_{600nm} of 0.2. Cell culture was passed through the channels at a shear force of 3Pa. Experiments were performed at 30°C. After 30 minutes of flow time the number of adhering cells was recorded. Adherent cells (stationary cells attached to the surface) were recorded by photographing four set locations along the flow channel. Channels were visualized using a Nikon Eclipse TS100 microscope and photographs were taken using a QICAM (QImaging) camera (Fast1394). Experiments were performed at n=4.

In vitro Biofilm Assays

Strains were grown in YPD overnight at 30°C. Overnight cultures were diluted to an OD_{600nm} = 0.5 in 2 ml of Spider medium in a sterile 12-well plate containing a silicone square (Bentec Medical Inc) that had been pre-treated with fetal bovine serum overnight and washed with PBS. The plate was incubated for 90 mins at 60 rpm at 37°C to allow initial cell adherence. Squares were washed in 1 ml PBS to remove un-adhered cells and then placed in a new 12 well plate containing 2 ml of Spider medium. The plate was incubated at 37°C at 60 rpm for 24 or 48hours.

Biofilm Biomass Assays

Silicone squares were weighed prior to in vitro biofilm assay setup. Biofilms were grown for 48 hrs (as per In vitro biofilm assay method above). The silicone squares were removed from wells, dried overnight at 37°C and re-weighed. Total biomass was calculated by subtracting the weight of the silicone prior to biofilm growth from the weight of the silicone after biofilm growth. The average biomass for each strain was calculated using independent samples and normalized to that of an un-inoculated silicone square.

Caspofungin Sensitivity Assays

Strains were grown overnight in YPD medium at 30°C with shaking. Cells were diluted to an OD_{600nm} of 3 in H_2O. Five-fold dilutions were made and plated on YPD control plates and on YPD containing 125 ng/ml caspofungin. Plates were incubated at 30°C for 24–48 hours and photographed.

Scanning Electron Microscopy

Biofilms were setup in 48 well plates ensuring channels between wells had carefully been constructed. The biofilm protocol was as per the in vitro biofilm assays methodology. Growth medium was removed from the well taking care not to remove the medium below the level of the biofilm. A fixative containing 2% glutaraldehyde and 0.1% ruthenium red, buffered with PBS was added. Cells were fixed for at least 1 hour. After primary fixation, the fixative was to the level of the biofilm, and the biofilm washed with at least 3 changes of PBS. As a secondary fixative, a 1% solution of OsO_4 buffered with PBS was carefully added. The biofilm was fixed for 1 hour, and washed with 3 changes of dH_2O. A continuous gradient from 100% dH_2O to 100% EtOH was produced and suspended in a syringe on a rack above a stir plate. A hot metal probe was used to cut a V-shaped drain into the side of the wells containing the biofilms. The 48 multiwell plate was placed at an angle into a lid of a 150 mm Petri dish. A needle, connected to a tube, attached to the syringe containing the gradient, was placed into the adjacent connected well. A small stir rod was placed in the adjacent connected well, and the stir plate was turned on. The valve of the gradient was opened, and the content of the gradient was slowly introduced into the adjacent connected well. The gradient diffused into the well with the biofilm, and exited through the v-shaped drain into the 150 mm Petri dish lid. After the gradient was delivered, several mls of 100% EtOH was added to the syringe and delivered to the adjacent connected well. After complete dehydration, the biofilm and substrate were dried in a Pelco CPD2 critical point dryer using CO_2, at 1200 psi, and 42°C. Dried biofilms were attached to SEM stubs. For cross section views, a sharp razor blade was used to cut the biofilm, and scrape away the biofilm from the foreground. The biofilms were coated with gold using a Pelco SC-6 sputter coater, and were examined using a Hitachi 2460N Scanning Electron Microscope. Digital images were obtained using Quartz PCI Image management system software.

NanoString analysis of gene expression

bcr1Δ/bcr1Δ and bcr*1Δ/bcr1Δtpk1Δ/tpk1Δ* strains were grown in Spider medium for 8 hours at 37°C with shaking. Samples containing 100 ng of *C. albicans* total RNA were mixed with custom designed probe code set and incubated at 65°C overnight (12–18 hours). The hybridized samples were processed on a nanoString prep station using the manufacturer's default program. The resultant cartridges were then transferred to the nanoString digital analyzer and scanned for 600 fields per sample. The raw counts were first adjusted for technical variability using the positive and negative controls of irrelevant RNA sequences included in the code set. The technically adjusted counts were then normalized for total input *C. albicans* RNA using *TDH3*. The normalized counts were used to compare gene expression levels among different samples.

Transmission Electron Microscopy

Cells were fixed for at least 1 hour in 2% glutaraldehyde, and 0.1% ruthenium red solution buffered with PBS. After washing in 3 changes of PBS, samples were placed in a 1% osmium tetroxide solution buffered with PBS for 1 hour. The cells were dehydrated using ethanol. The dehydration was in 5-minute steps at concentrations of 50%, 70%, 95%, and 3 10-minute steps of 100% ethanol. Propylene oxide was used as a transitional solvent. The propylene oxide was changed twice, at 10 minutes for each change. The sample was placed in a 1:2 mixture of Spurr's resin and propylene oxide, and held at room temperature overnight. The next day the sample was transferred to a 1:1 mixture of Spurr's resin and propylene oxide and held at room temperature. After 8 hours, the mixture was changed to a 1:2 mixture of Spurr's resin and propylene oxide, and held with the tubes open, at room temperature, overnight in a desiccator. The Spurr's resin and propylene oxide mixture was removed and replaced with 100% Spurr's resin. The sample was infiltrated with the resin for

an additional 8 hours, placed in embedding molds, and polymerized for 24 hours at 60°C. Thin sections were cut using a Reichert-Jung Ultracut E ultramicrotome and a DDK Diamond knife. Thin sections on copper grids were stained with 1% uranyl acetate and Reynold's lead citrate. Sections were viewed on a Hitachi H-7100 TEM transmission electron microscope. Digital images were obtained using AMT Advantage 10 CCD Camera and NIH Image software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to members of the 2008 Molecular Mycology Class at the Marine Biological Laboratory for preparation of RNA for initial microarray experiments. We are grateful to Tatyana Aleynikova for preparation and management of laboratory stocks and supplies, and to Jonathan Finkel for his work on biofilm SEM imaging. This work was funded by NIH research grant R01 AI067703 (APM), a National University of Ireland Travelling Studentship (SF), and by support from the Canadian Institutes of Health Research (AN).

References

Bahn YS, Sundstrom P. CAP1, an adenylate cyclase-associated protein gene, regulates bud-hypha transitions, filamentous growth, and cyclic AMP levels and is required for virulence of Candida albicans. J Bacteriol. 2001; 183:3211–3223. [PubMed: 11325951]

Bimbo A, Jia Y, Poh SL, Karuturi RK, den Elzen N, Peng X, Zheng L, O'Connell M, Liu ET, Balasubramanian MK, Liu J. Systematic deletion analysis of fission yeast protein kinases. Eukaryot Cell. 2005; 4:799–813. [PubMed: 15821139]

Biswas S, Van Dijck P, Datta A. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of Candida albicans. Microbiol Mol Biol Rev. 2007; 71:348–376. [PubMed: 17554048]

Blankenship JR, Fanning S, Hamaker JJ, Mitchell AP. An extensive circuitry for cell wall regulation in Candida albicans. PLoS Pathog. 2010; 6:e1000752. [PubMed: 20140194]

Bockmuhl DP, Ernst JF. A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of Candida albicans. Genetics. 2001; 157:1523–1530. [PubMed: 11290709]

Bockmuhl DP, Krishnamurthy S, Gerads M, Sonneborn A, Ernst JF. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of Candida albicans. Mol Microbiol. 2001; 42:1243–1257. [PubMed: 11886556]

Bruno VM, Kalachikov S, Subaran R, Nobile CJ, Kyratsous C, Mitchell AP. Control of the C. albicans cell wall damage response by transcriptional regulator Cas5. PLoS Pathog. 2006; 2:e21. [PubMed: 16552442]

Cassola A, Parrot M, Silberstein S, Magee BB, Passeron S, Giasson L, Cantore ML. Candida albicans lacking the gene encoding the regulatory subunit of protein kinase A displays a defect in hyphal formation and an altered localization of the catalytic subunit. Eukaryot Cell. 2004; 3:190–199. [PubMed: 14871949]

Cloutier M, Castilla R, Bolduc N, Zelada A, Martineau P, Bouillon M, Magee BB, Passeron S, Giasson L, Cantore ML. The two isoforms of the cAMP-dependent protein kinase catalytic subunit are involved in the control of dimorphism in the human fungal pathogen Candida albicans. Fungal Genet Biol. 2003; 38:133–141. [PubMed: 12553943]

D'Souza CA, Heitman J. Conserved cAMP signaling cascades regulate fungal development and virulence. FEMS Microbiol Rev. 2001; 25:349–364. [PubMed: 11348689]

Dwivedi P, Thompson A, Xie Z, Kashleva H, Ganguly S, Mitchell AP, Dongari-Bagtzoglou A. Role of Bcr1-activated genes Hwp1 and Hyr1 in Candida albicans oral mucosal biofilms and neutrophil evasion. PLoS One. 2011; 6:e16218. [PubMed: 21283544]

Finkel JS, Mitchell AP. Genetic control of Candida albicans biofilm development. Nat Rev Microbiol. 2011; 9:109–118. [PubMed: 21189476]

Finkel JS, Xu W, Huang D, Hill EM, Desai JV, Woolford CA, Nett JE, Taff H, Norice CT, Andes DR, Lanni F, Mitchell AP. Portrait of Candida albicans Adherence Regulators. PLoS Pathog. 2012; 8:e1002525. [PubMed: 22359502]

Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, Fell HP, Ferree S, George RD, Grogan T, James JJ, Maysuria M, Mitton JD, Oliveri P, Osborn JL, Peng T, Ratcliffe AL, Webster PJ, Davidson EH, Hood L, Dimitrov K. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008; 26:317–325. [PubMed: 18278033]

Giacometti R, Kronberg F, Biondi RM, Passeron S. Catalytic isoforms Tpk1 and Tpk2 of Candida albicans PKA have non-redundant roles in stress response and glycogen storage. Yeast. 2009; 26:273–285. [PubMed: 19391100]

Giacometti R, Kronberg F, Biondi RM, Passeron S. Candida albicans Tpk1p and Tpk2p isoforms differentially regulate pseudohyphal development, biofilm structure, cell aggregation and adhesins expression. Yeast. 2011; 28:293–308. [PubMed: 21456055]

Green CB, Cheng G, Chandra J, Mukherjee P, Ghannoum MA, Hoyer LL. RT-PCR detection of Candida albicans ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. Microbiology. 2004; 150:267–275. [PubMed: 14766904]

Gregori C, Glaser W, Frohner IE, Reinoso-Martin C, Rupp S, Schuller C, Kuchler K. Efg1 Controls caspofungin-induced cell aggregation of Candida albicans through the adhesin Als1. Eukaryot Cell. 2011; 10:1694–1704. [PubMed: 22037180]

Harcus D, Nantel A, Marcil A, Rigby T, Whiteway M. Transcription profiling of cyclic AMP signaling in Candida albicans. Mol Biol Cell. 2004; 15:4490–4499. [PubMed: 15269278]

Hogan DA, Muhlschlegel FA. Candida albicans developmental regulation: adenylyl cyclase as a coincidence detector of parallel signals. Curr Opin Microbiol. 2011; 14:682–686. [PubMed: 22014725]

Hoyer LL, Green CB, Oh SH, Zhao X. Discovering the secrets of the Candida albicans agglutinin-like sequence (ALS) gene family--a sticky pursuit. Med Mycol. 2008; 46:1–15. [PubMed: 17852717]

Kamai Y, Kubota M, Hosokawa T, Fukuoka T, Filler SG. Contribution of Candida albicans ALS1 to the pathogenesis of experimental oropharyngeal candidiasis. Infect Immun. 2002; 70:5256–5258. [PubMed: 12183577]

Leberer E, Harcus D, Dignard D, Johnson L, Ushinsky S, Thomas DY, Schroppel K. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus Candida albicans. Mol Microbiol. 2001; 42:673–687. [PubMed: 11722734]

Ma H, Kunes S, Schatz PJ, Botstein D. Plasmid construction by homologous recombination in yeast. Gene. 1987; 58:201–216. [PubMed: 2828185]

Nantel, A.; Rigby, T.; Hogues, H.; Whiteway, M. Microarrays for studying pathogenicity in Candida albicans. In: Kavanaugh, K., editor. Medical mycology: cellular and molecular techniques. Hoboken (New Jersey): Wiley Press; 2006. p. 181-210.

Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan QT, Edwards JE, Filler SG, Mitchell AP. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog. 2006a; 2:e63. [PubMed: 16839200]

Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, Johnson AD. A recently evolved transcriptional network controls biofilm development in Candida albicans. Cell. 2012; 148:126–138. [PubMed: 22265407]

Nobile CJ, Mitchell AP. Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr Biol. 2005; 15:1150–1155. [PubMed: 15964282]

Nobile CJ, Nett JE, Andes DR, Mitchell AP. Function of Candida albicans adhesin Hwp1 in biofilm formation. Eukaryot Cell. 2006b; 5:1604–1610. [PubMed: 17030992]

Nobile CJ, Schneider HA, Nett JE, Sheppard DC, Filler SG, Andes DR, Mitchell AP. Complementary adhesin function in C. albicans biofilm formation. Curr Biol. 2008a; 18:1017–1024. [PubMed: 18635358]

Nobile CJ, Solis N, Myers CL, Fay AJ, Deneault JS, Nantel A, Mitchell AP, Filler SG. Candida albicans transcription factor Rim101 mediates pathogenic interactions through cell wall functions. Cell Microbiol. 2008b; 10:2180–2196. [PubMed: 18627379]

Park G, Servin JA, Turner GE, Altamirano L, Colot HV, Collopy P, Litvinkova L, Li L, Jones CA, Diala FG, Dunlap JC, Borkovich KA. Global analysis of serine-threonine protein kinase genes in Neurospora crassa. Eukaryot Cell. 2011; 10:1553–1564. [PubMed: 21965514]

Park H, Myers CL, Sheppard DC, Phan QT, Sanchez AA, EEJ, Filler SG. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell Microbiol. 2005; 7:499–510. [PubMed: 15760450]

Pitarch A, Sanchez M, Nombela C, Gil C. Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic fungus Candida albicans cell wall proteome. Mol Cell Proteomics. 2002; 1:967–982. [PubMed: 12543933]

Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, Gow NA, Gaillardin C, Munro CA, Richard ML. Functional analysis of Candida albicans GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity. Fungal Genet Biol. 2008; 45:1404–1414. [PubMed: 18765290]

Reuss O, Vik A, Kolter R, Morschhauser J. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene. 2004; 341:119–127. [PubMed: 15474295]

Roth FP, Lipshitz HD, Andrews BJ. Q&A: epistasis. J Biol. 2009; 8:35. [PubMed: 19486505]

Sentandreu M, Nieto A, Iborra A, Elorza MV, Ponton J, Fonzi WA, Sentandreu R. Cloning and characterization of CSP37, a novel gene encoding a putative membrane protein of Candida albicans. J Bacteriol. 1997; 179:4654–4663. [PubMed: 9244249]

Singleton DR, Masuoka J, Hazen KC. Cloning and analysis of a Candida albicans gene that affects cell surface hydrophobicity. J Bacteriol. 2001; 183:3582–3588. [PubMed: 11371521]

Sonneborn A, Bockmuhl DP, Gerads M, Kurpanek K, Sanglard D, Ernst JF. Protein kinase A encoded by TPK2 regulates dimorphism of Candida albicans. Mol Microbiol. 2000; 35:386–396. [PubMed: 10652099]

Souto G, Giacometti R, Silberstein S, Giasson L, Cantore ML, Passeron S. Expression of TPK1 and TPK2 genes encoding PKA catalytic subunits during growth and morphogenesis in Candida albicans. Yeast. 2006; 23:591–603. [PubMed: 16823887]

Spellberg BJ, Ibrahim AS, Avenissian V, Filler SG, Myers CL, Fu Y, Edwards JE Jr. The anti-Candida albicans vaccine composed of the recombinant N terminus of Als1p reduces fungal burden and improves survival in both immunocompetent and immunocompromised mice. Infect Immun. 2005; 73:6191–6193. [PubMed: 16113347]

Stoldt VR, Sonneborn A, Leuker CE, Ernst JF. Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. 1997; 16:1982–1991. [PubMed: 9155024]

Teste MA, Enjalbert B, Parrou JL, Francois JM. The Saccharomyces cerevisiae YPR184w gene encodes the glycogen debranching enzyme. FEMS Microbiol Lett. 2000; 193:105–110. [PubMed: 11094287]

Urban C, Sohn K, Lottspeich F, Brunner H, Rupp S. Identification of cell surface determinants in Candida albicans reveals Tsa1p, a protein differentially localized in the cell. FEBS Lett. 2003; 544:228–235. [PubMed: 12782322]

Wilson RB, Davis D, Mitchell AP. Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. J Bacteriol. 1999; 181:1868–1874. [PubMed: 10074081]

Wu X, Hart H, Cheng C, Roach PJ, Tatchell K. Characterization of Gac1p, a regulatory subunit of protein phosphatase type I involved in glycogen accumulation in Saccharomyces cerevisiae. Mol Genet Genomics. 2001; 265:622–635. [PubMed: 11459182]

Zhao X, Oh SH, Cheng G, Green CB, Nuessen JA, Yeater K, Leng RP, Brown AJ, Hoyer LL. ALS3 and ALS8 represent a single locus that encodes a Candida albicans adhesin; functional comparisons between Als3p and Als1p. Microbiology. 2004; 150:2415–2428. [PubMed: 15256583]

Zhao X, Oh SH, Yeater KM, Hoyer LL. Analysis of the Candida albicans Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. Microbiology. 2005; 151:1619–1630. [PubMed: 15870470]

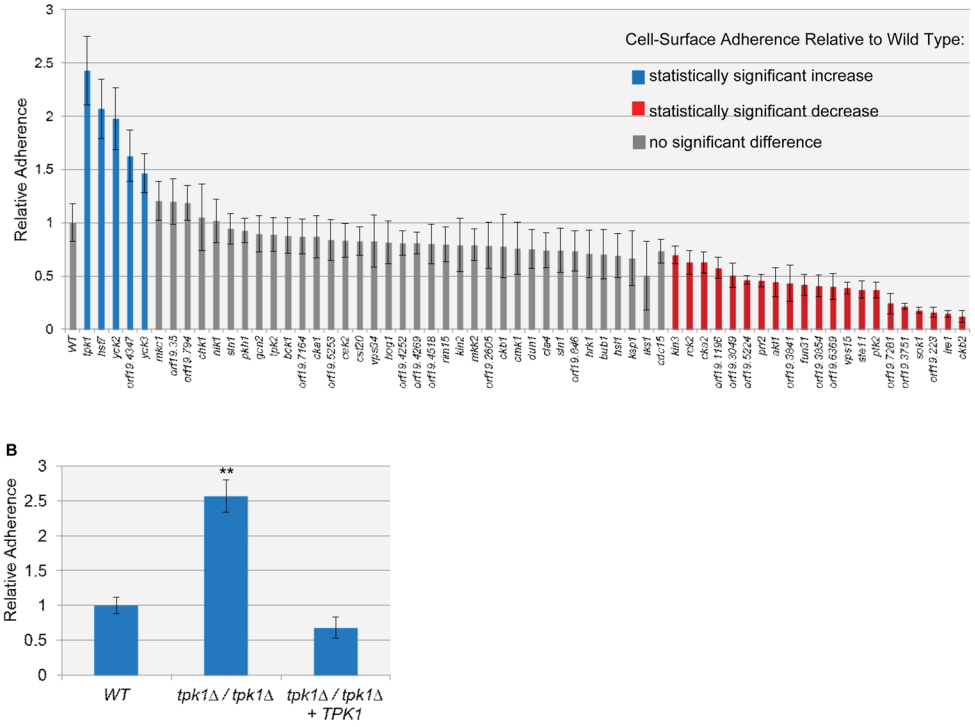

Figure 1. Cell-surface adherence of *C. albicans* PK mutants

(A) Adherence to silicone was measured using the Fluxion flow assay. The first bar on the graph represents wild-type level of adherence. Error bars represent standard deviation values. Mutants that filamented, had severe growth defects or aggregated were not assayed; these included homozygous insertion mutants for *PBS2*, *GIN4*, *Orf19.1874*, *IME2*, *KIS1*, *SWE1*, and *CBK1*. The wild-type strain is DAY286. (B) Adherence to silicone of wild type, *tpk1Δ/tpk1Δ* and *tpk1Δ/tpk1Δ+TPK1Δ* strains was measured using the Fluxion flow assay. ** denotes p=0.0011 for wild type versus *tpk1Δ/tpk1Δ*. The strains used were DAY185, SF1130, and SF1119.

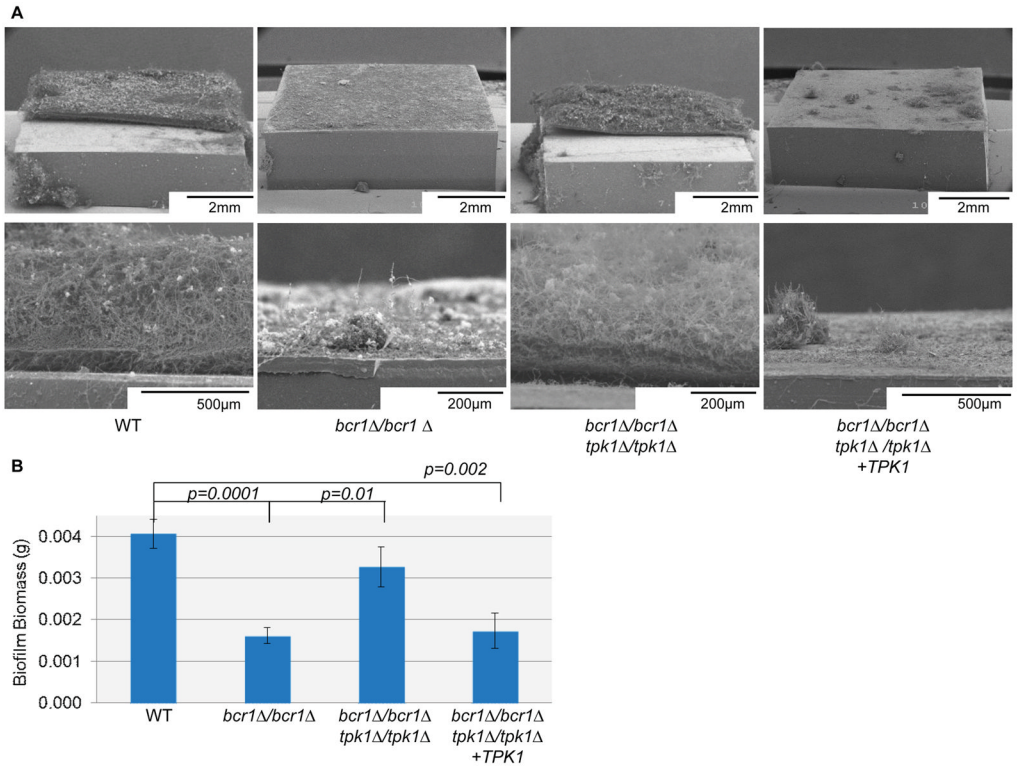

Figure 2. Relationship between Tpk1 and Bcr1 in biofilm formation

(A) Apical and cross-sectional views of biofilms grown on silicone squares for 2 days at 37°C in Spider medium, visualized by scanning electron microscopy. (B) Biofilm biomass measurements. Strain genotypes are given beneath each image. The strains used were DAY185, CJN702, SF1151, and SF1147.

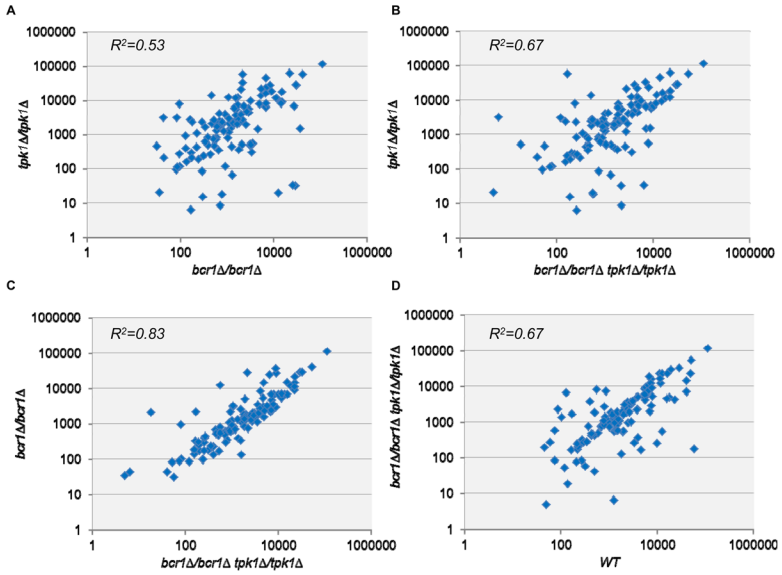

Figure 3. Relationship between Tpk1 and Bcr1 in gene expression

Expression data was generated by nanoString measurement (Table S1) using probes for 117 genes. RNA levels for each gene were normalized to internal control *TDH3* RNA levels, and the mean of three biological replicates for each gene was plotted here. (A) Normalized gene expression in a *bcr1Δ/bcr1Δ* strain (x-axis) versus a *tpk1Δ/tpk1* strain (y-axis). (B) Normalized gene expression in a *bcr1Δ/bcr1Δ tpk1Δ/tpk1* strain (x-axis) versus a *tpk1Δ/tpk1* strain (y-axis). (C) Normalized gene expression in a *bcr1Δ/bcr1Δ tpk1Δ/tpk1* strain (x-axis) versus a *bcr1Δ/bcr1Δ* strain (y-axis). (D) Normalized gene expression in a wild-type strain (x-axis) versus a *bcr1Δ/bcr1Δ tpk1Δ/tpk1* strain (y-axis). The strains used were DAY185, CJN702, SF1151, and SF1130.

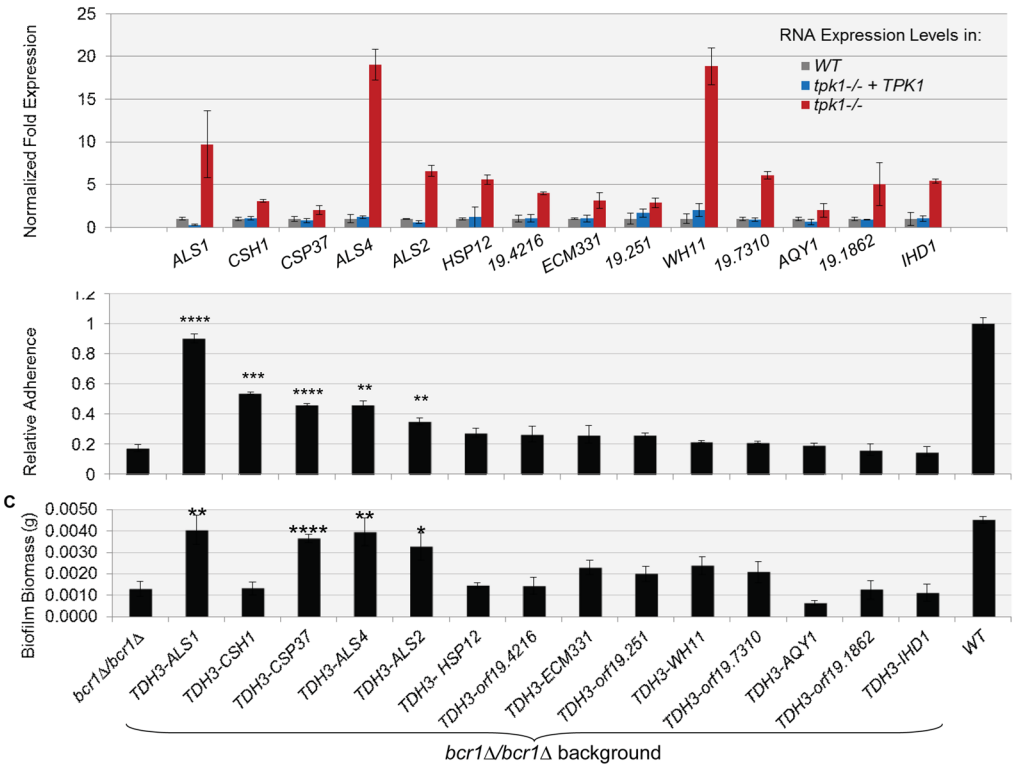

Figure 4. Effect of Tpk1 target genes on adherence and biofilm formation in a *bcr1Δ/ bcr1Δ* background

(A) Gene expression measurements for the 14 most highly Tpk1-repressed identified by microarray analysis. The bar graph shows results of QRTPCR assays that were conducted in triplicate samples of the wild type, complemented, and mutant strains. The strains used were DAY286, JJH384, and JJH233. (B and C) Each of the most highly Tpk1-repressed genes was overexpressed from the *TDH3* promoter in a *bcf1Δ/bcr1Δ* background, and strains were assayed for (B) adherence, using a Fluxion flow assay, and (C) biofilm biomass. Error bars represent standard deviation. Statistical significance is relative to *bcr1Δ/bcr1Δ*. *p≤0.05, **p≤0.005, ***p≤0.001, ***p≤0.0005. The strains used were DAY185, CJN702, SF1255, SF1258, SF1303, SF1294, SF1259, SF1300, SF1284, SF1264, SF1253, SF1275, SF1257, SF1297, and SF1295.

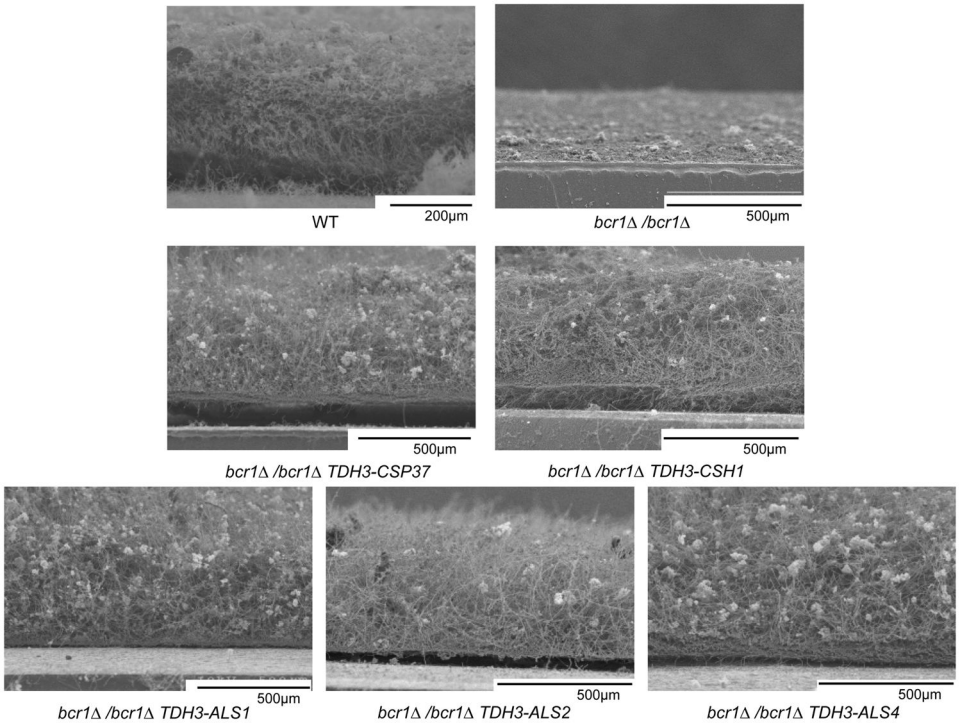

Figure 5. Structure of biofilms of *bcr1Δ/bcr1Δ* strains that overexpress Tpk1-repressed genes

Biofilms on silicone squares were visualized in cross sectional views by scanning electron microscopy. Genotypes are given beneath each panel. The strains used were DAY185, CJN702, SF1258, SF1255, SF1271, SF1294, and SF1303.

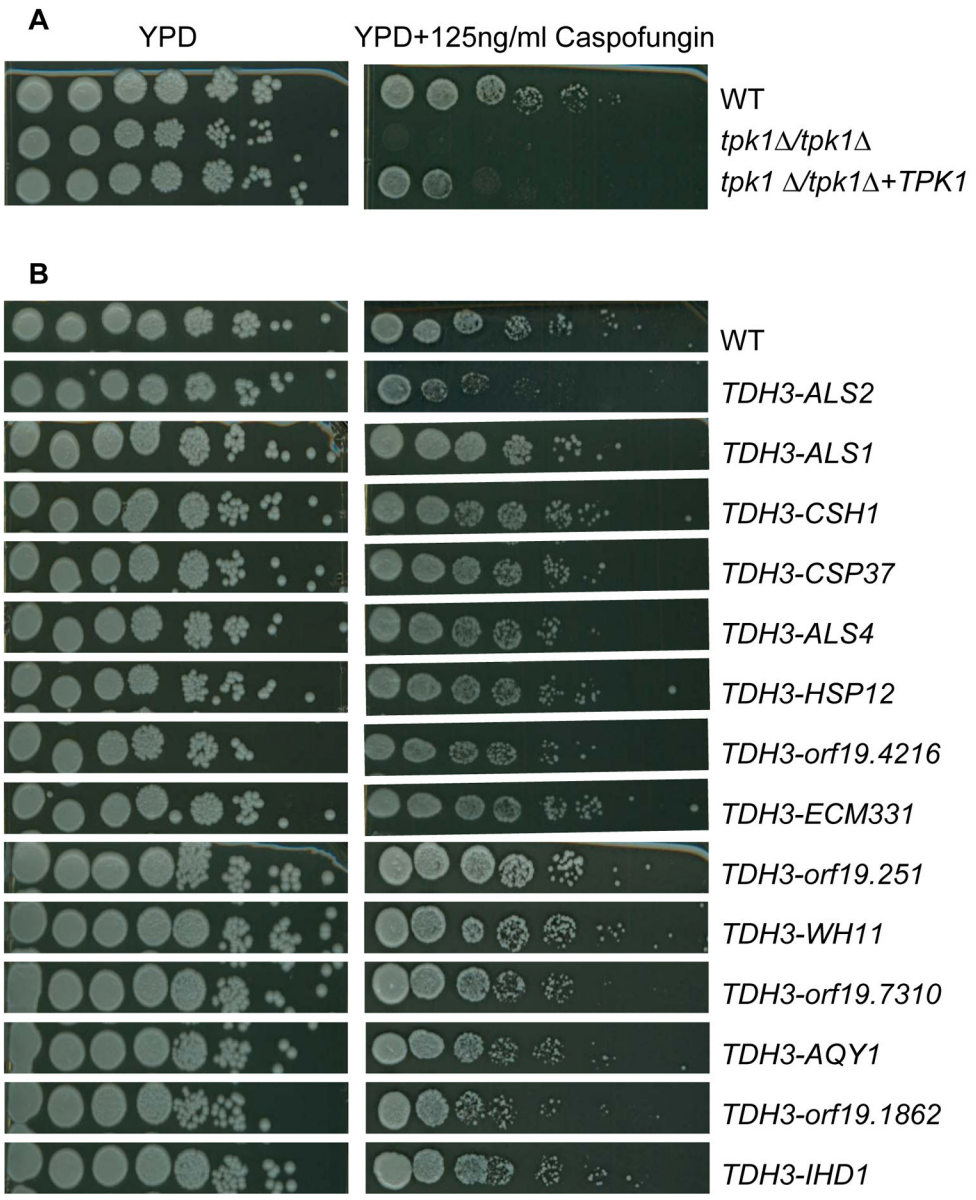

Figure 6. Role of Tpk1 and Tpk1-repressed genes in cell wall integrity

Serial spot-dilution assays were used to compare growth of strains on control YPD (left panels) and YPD + caspofungin (right panels). (A) Comparison of wild-type (DAY185), *tpk1Δ/tpk1Δ* mutant, and *tpk1Δ/tpk1Δ+TPK1* complemented strains in the BWP17 background. The strains used were DAY185, SF1130, and SF1119. (B) Comparison of wild-type strain SC5314 and its derivatives that overexpress individual Tpk1-repressed genes. The strains used were SC5314, SF1127, SF1190, SF1160, SF1204, SF1129, SF1242, SF1306, SF1280, SF1236, SF1161, SF1279, SF1245, SF1290, and SF1265.

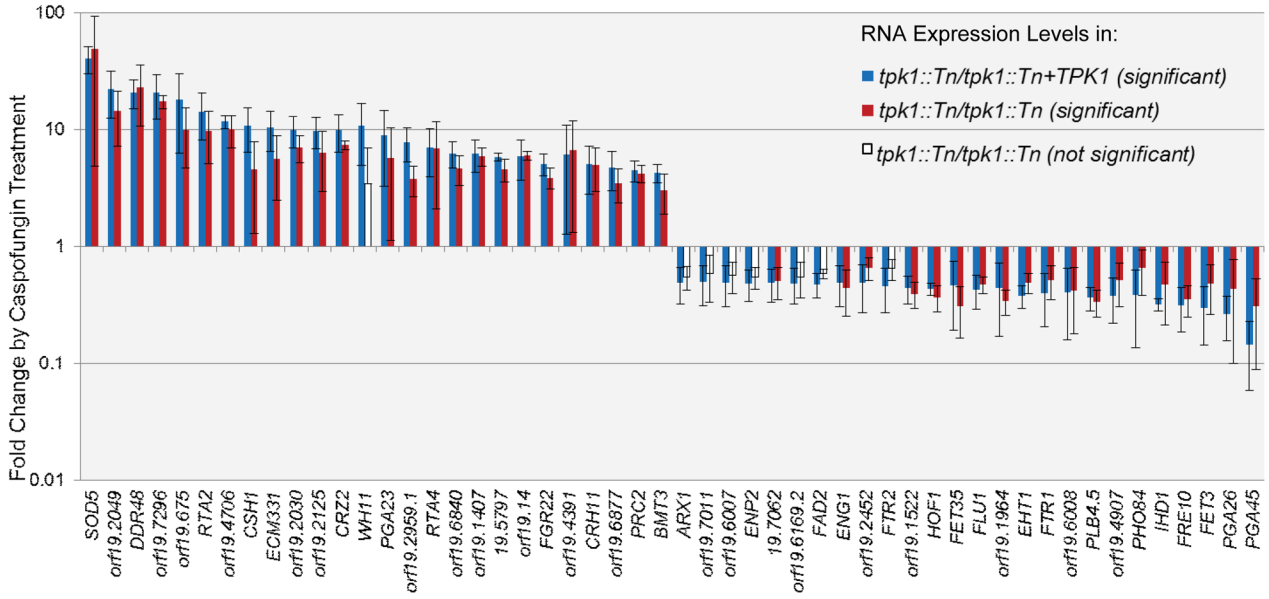

Figure 7. Caspofungin-responsive gene expression in *tpk1::Tn/tpk1::Tn* mutant and *tpk1::Tn/tpk1::Tn+TPK1* complemented strains

Microarray analysis was used to identify the most highly caspofungin-responsive genes in the *tpk1::Tn/tpk1::Tn+TPK1* complemented strain. The fold change, relative to expression in the absence of caspofungin, is presented for both the *tpk1::Tn/tpk1::Tn* mutant and *tpk1::Tn/tpk1::Tn+TPK1* complemented strains. The graph represents significant responses of the complement strain, significant responses of the mutant strain and responses in the mutant that are not statistically significant relative to untreated cells. The strains used were JJH384 and JJH233. Error bars indicate standard deviation values.

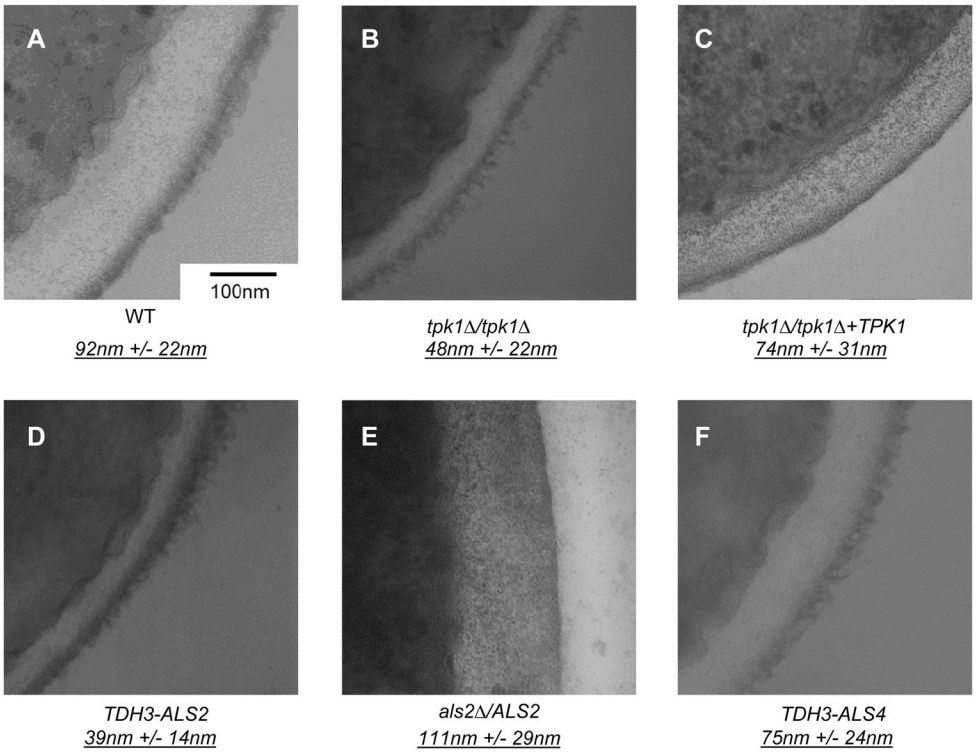

Figure 8. Cell wall depth visualization

Transmission electron microscopy of sectioned cells was used to determine cell wall thickness on 10 unselected cells of each strain, with means and standard deviations indicated. Representative images are shown. (A) The wild-type (SC5314) cell wall measurement was 92nm+/−22nm. (B) The *tpk1Δ/tpk1Δ* (SF1358) cell wall measurement was 48nm+/−22nm. p=<0.0001 relative to wild type. (C) The *tpk1Δ/tpk1Δ* +TPK1 (SF1119) cell wall measurement was 74nm+/−31nm. p=0.04 relative to *tpk1Δ/tpk1Δ* and not statistically significant (p=0.08) from wild type. (D) The wild-type strain overexpressing *ALS2* (SF1127) cell wall measurement was 39nm +/− 14nm. p=<0.0001 relative to wild-type. (E) A heterozygous *ALS2* deletion strain (SF1332) had a cell wall measurement of 111nm +/− 29nm. p=0.04 relative to wild type. (F) The wild-type strain overexpressing *ALS4* (SF1129) cell wall measurement was 75nm +/− 24nm. p= 0.06 relative to wild type.

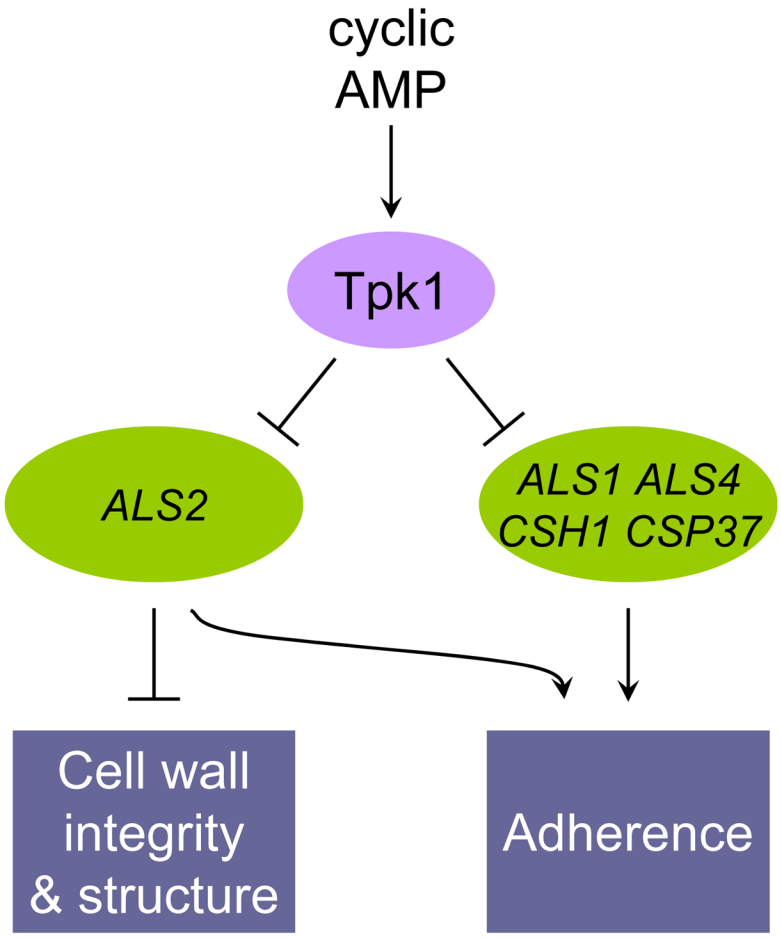

Figure 9. Model for control of cell wall functions by Tpk1

Tpk1, one of the cyclic AMP-dependent protein kinase catalytic subunits, governs adherence, cell wall integrity, and cell wall structure. Our findings argue that many of these roles are mediated by Tpk1-dependent repression of select target genes. The target genes *ALS1*, *ALS4*, *CSH1*, and *CSP37* act to promote adherence. The target gene *ALS2* acts to promote adherence and also governs cell wall integrity and structure.

Table 1

Strains used in this study

Strain	Genotype	Source/Ref
SC5314	Wild type clinical isolate	(Gillum *et al.*, 1984)
BWP17	*ura3Δ::λimm434 arg4::hisG his1::hisG* *ura3Δ::λimm434 arg4::hisG his1::hisG*	Mitchell Lab
DAY286	*ura3Δ::λiimm434 ARG4:URA3::arg4::hisG his1::hisG* *ura3Δ::λiimm434 arg4::hisG his1::hisG*	Mitchell Lab
DAY185	*ura3Δ::λiimm434 HIS1::his1::hisG ARG4::URA3::arg4::hisG* *ura3Δ::λiimm434 his1::hisG arg4::hisG*	Mitchell Lab
JJH384	*ura3Δ::λimm434 arg4::hisG his1::hisG tpk1::Tn7-UAU1* *ura3Δ::λimm434 arg4::hisG his1::hisG tpk1::Tn7-URA3*	(Blankenship et al., 2010)
JJH233	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1-TPK1 tpk1::Tn7-UAU1* *ura3Δ::λimm434 arg4::hisG his1::hisG tpk1::Tn7-URA3*	(Blankenship et al., 2010)
CJN702	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3*	(Nobile & Mitchell, 2005)
SF1130	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 tpk1::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG tpk1::URA3*	This Study
SF1119	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1-TPK1 tpk1::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG tpk1::URA3*	This Study
SF1358	*SC5314 - tpk1-1Δ::FRT/tpk1-2Δ::FRT*	This Study
SF1151	*ura3Δ::λimm434 arg4::hisG his1::hisG+pHIS1 bcr1Δ::dpl200 tpk1::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1Δ::dpl200 tpk1::URA3*	This Study
SF1147	*ura3Δ::λimm434 arg4::hisG his1::hisG+pHIS1-TPK1 bcr1Δ::dpl200 tpk1::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1Δ::dpl200 tpk1::URA3*	This Study
SF803	*ura3Δ::λimm434 arg4::hisG his1::hisG bcr1Δ::dpl200* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1Δ::dpl200*	This Study
SF1271	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 ALS1::pAgTEF1-NAT1-AgTEF1UTR-TDH3-ALS1* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 ALS1*	This study
SF1255	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 CSH1::pAgTEF1-NAT1-AgTEF1UTR-TDH3- CSH1* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 CSH1*	This study
SF1258	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 CSP37::pAgTEF1-NAT1-AgTEF1UTR-TDH3- CSP37* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 CSP37*	This study
SF1303	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 ALS4::pAgTEF1-NAT1-AgTEF1UTR-TDH3-ALS4* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 ALS4*	This study
SF1294	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 ALS2::pAgTEF1-NAT1-AgTEF1UTR-TDH3-ALS2* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 ALS2*	This study
SF1259	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 HSP12::pAgTEF1-NAT1-AgTEF1UTR-TDH3- HSP12* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 HSP12*	This study
SF1300	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 orf19.4216::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.4216* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 orf19.4216*	This study
SF1284	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 ECM331::pAgTEF1-NAT1-AgTEF1UTR-TDH3- ECM331* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 ECM331*	This study
SF1264	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 orf19.251::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.251* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 orf19.251*	This study
SF1253	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 WH11::pAgTEF1-NAT1-AgTEF1UTR-TDH3- WH11* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 WH11*	This study
SF1275	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 orf19.7310::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.7310* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 orf19.7310*	This study
SF1257	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 AQY1::pAgTEF1-NAT1-AgTEF1UTR-TDH3- AQY1* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 AQY1*	This study
SF1297	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 orf19.1862::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.1862* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 orf19.1862*	This study
SF1295	*ura3Δ::λimm434 arg4::hisG his1::hisG::pHIS1 bcr1::ARG4 IHD1::pAgTEF1-NAT1-AgTEF1UTR-TDH3- IHD1* *ura3Δ::λimm434 arg4::hisG his1::hisG bcr1::URA3 IHD1*	This study
SF1190	*SC5314 - ALS1::pAgTEF1-NAT1-AgTEF1UTR-TDH3-ALS1* *ALS1*	This study
SF1160	*SC5314 - CSH1::pAgTEF1-NAT1-AgTEF1UTR-TDH3-CSH1* *CSH1*	This study

SF1204	*SC5314 - CSP37::pAgTEF1-NAT1-AgTEF1UTR-TDH3-CSP37* *CSP37*	This study
SF1129	*SC5314 - ALS4::pAgTEF1-NAT1-AgTEF1UTR-TDH3-ALS4* *ALS4*	This study
SF1127	*SC5314 - ALS2::pAgTEF1-NAT1-AgTEF1UTR-TDH3-ALS2* *ALS2*	This study
SF1242	*SC5314 - HSP12::pAgTEF1-NAT1-AgTEF1UTR-TDH3- HSP12* *HSP12*	This study
SF1306	*SC5314 - orf19.4216::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.4216* *orf19.4216*	This study
SF1280	*SC5314 - ECM331::pAgTEF1-NAT1-AgTEF1UTR-TDH3- ECM331* *ECM331*	This study
SF1236	*SC5314 - orf19.251::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.251* *orf19.251*	This study
SF1161	*SC5314 - WH11::pAgTEF1-NAT1-AgTEF1UTR-TDH3- WH11* *WH11*	This study
SF1279	*SC5314 - orf19.7310::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.7310* *orf19.7310*	This study
SF1245	*SC5314 - AQY1::pAgTEF1-NAT1-AgTEF1UTR-TDH3- AQY1* *AQY1*	This study
SF1290	*SC5314 - orf19.1862::pAgTEF1-NAT1-AgTEF1UTR-TDH3- orf19.1862* *orf19.1862*	This study
SF1265	*SC5314 - IHD1::pAgTEF1-NAT1-AgTEF1UTR-TDH3- IHD1* *IHD1*	This study
SF1349	*ura3Δ::λimm434 arg4::hisG his1::hisG als2::ARG4* *ura3Δ::λimm434 arg4::hisG his1::hisG ALS2*	This study
SF1332	*ura3Δ::λimm434 arg4::hisG his1::hisG als2::URA3* *ura3Δ::λimm434 arg4::hisG his1::hisG ALS2*	This study
CAI4	*Δura3::imm434/Δura3::imm434*	(Fonzi & Irwin, 1993)
IIHB6	*As CAI4 but tpk1D::hisG-URA3-hisG//tpk1D::hisG*	(Bockmuhl et al., 2001)
HPY300U	*tpk1Δ::hisG/tpkΔ1::hisG ura3Δ::imm434/ura3Δ::imm434::URA3*	(Park et al., 2005)
HPY321	*tpk1Δ::hisG/tpk1Δ::hisG::TPK-dpl200 ura3Δ::imm434/ura3Δ::imm434::URA3*	(Park et al., 2005)

Table 2

Primers used in this study

Primer Name	Primer Sequence	
TPK1-5DR	AGATCAATATATCCTATCGTTATCCTCCTCCTTCTCCTTTCAACTTTTGAAAAAGGTGATATTATTCAACACTGTTTCTGTTTTATCAACCAAAACCAGGTTTCCCAGTCACGACGTT	This study
TPK1-3DR	TCTTTTTTCTTGTTCTTTCTTGCAAATTAATTATACATCTATAAAACTAGTTATCATAATTAACATTGTTGTGCCAATAAATACAATTTTATTTTTACATGTGGAATTGTGAGCGGATA	This study
TDH3F	ATCCCACAAGGACTGGAGA	Blankenship *et al.*, 2010
TDH3R	GCAGAAGCTTTAGCAACGTG	Blankenship *et al.*, 2010
ALS1F	GACTAGTGAACCAACAAATACCAGA	Green *et al.*, 2004
ALS1R	CCAGAAGAAACAGCAGGTGA	Green *et al.*, 2004
ALS2F	CCAAGTATTAACAAAGTTTCAATCACTTAT	Green *et al.*, 2004
ALS2R	TCTCAATCTTAAATTGAACGGCTTAC	Green *et al.*, 2004
ALS3F	CCACTTCACAATCCCCATC	Green *et al.*, 2004
ALS3R	CAGCAGTAGTAGTAACAGTAGTAGTTTCATC	Green *et al.*, 2004
ALS4F	CCCAGTCTTTCACAAGCAGTAAAT	Green *et al.*, 2004
ALS4R	GTAAATGAGTCATCAACAGAAGCC	Green *et al.*, 2004
ALS5F	TGACTACTTCCAGATTTATGCCGAG	Green *et al.*, 2004
ALS5R	ATTGATACTGGTTATTATCTGAGGGAGAAA	Green *et al.*, 2004
ALS6F	GACTCCACAATCATCTAGTAGCTTGGTTT	Green *et al.*, 2004
ALS6R	CAATTGTCACATCATCTTTTGTTGC	Green *et al.*, 2004
ALS7F	GAAGAGAACTAGCGTTTGGTCTAGTTGT	Green *et al.*, 2004
ALS7R	TGGCATACTCCAATCATTTATTTCA	Green *et al.*, 2004
ALS9F	CCATATTCAGAAACAAAGGGTTC	Green *et al.*, 2004
ALS9R	AACTGAAACTGCTGGATTTGG	Green *et al.*, 2004
ORF19.251F	GTCATGGTCCTGCCATTTTT	This study
ORF19.251R	AGCACCTTCCTTTTCAGCAA	This study
ORF19.4477F	TGGTGTTTTGTGTCGTCCAT	This study
ORF19.4477R	CACCATGCCAATGAAACTTG	This study
ORF19.7310F	TCAGCAACAAGGTTGACGAG	This study
ORF19.7310R	GAGTAGCCTGAGACCCAACG	This study
ORF19.1862F	ACAACGTGGTGCTGAAGGTA	This study

Primer Name	Primer Sequence	
ORF19.1862R	GTGGGATCCACCAATTTCAC	This study
ORF19.5760F	TGATGCCTCAGGTACTGCTG	This study
ORF19.5760R	CCTGAAGCAGCAGTTGTCAT	This study
ORF19.3160F	AAAGGGCAAGGAACAAGTCA	This study
ORF19.3160R	TTCAGCAGCCTTTCCAATTT	This study
ORF19.3548.1F	TTGGTGATAAAATCGAATCCAA	This study
ORF19.3548.1R	GCATCAGAAGCTTTTTGGACA	This study
ORF19.2531F	TGAAAAATGACGCCGATAAA	This study
ORF19.2531R	TCGTAATGTTTGTGCCATTCA	This study
ORF19.4216F	AAAGGGCAAGGAACAAGTCA	This study
ORF19.4216R	TTCAGCAGCCTTTCCAATTT	This study
ORF19.2849F	TTGCTAACCAAGACCCAACC	This study
ORF19.2849R	AATTGGTGGGACAGCTTGAG	This study
ECM331F	CCAAACTTGAAATCCGTTGG	This study
ECM331R	TGGACCAGAAATGGTGACAG	This study
AQY1F	TTGCTAACCAAGACCCAACC	This study
AQY1R	AATTGGTGGGACAGCTTGAG	This study
HSP12-O/EXP-F	GTGATAATAAGTGTGAAGAGTAGTAATTGAAGTTTGGTGTGTCTAGGTGGTGTTTAAGACTGTTTAGTATTTTCGCAAGAATTTTGTTAACTTTGTGTGTATCAAGCTTGCCTCGTCCCC	This study
HSP12-O/EXP-R	TGGTGTTTGTCGAAAAAAAACAAGATTCAAAGAATTTACCTCTACTAGAGAAATTGACTCTATTTATATTGTTTTTCGAGAGGGGGGCAAAGGTTGCCATATTTGAATTCAATTGTGATG	This study
ORF19.7310-O/EXP-F	CCTTGGTTTTCGGACATGAAAATTGATACTAATAAGTGTGTTTTTATTACCAAACCCAGTAAAAGTTGCAAATACAATTCTCATCAAAGTTTCTTTGAGAATCAAGCTTGCCTCGTCCCC	This study
ORF19.7310-O/EXP-R	AAAATTGTTTTAAATCATCATTTGTCCATTGATCAAAACCATAATTGGCAACCACAAAAGTGATAGATACATATAACCATACAATGATTGGGAAATACATATTTGAATTCAATTGTGATG	This study
IHD1-O/EXP-F	CTATTAAATTGACCCCATTACTACATAGATTTTTAGTTCATAGTCTTTATGGTTTGCTTTGTTTCTTTAATCGCATTGATAATTCAATATTTGTGTTACAATCAAGCTTGCCTCGTCCCC	This study
IHD1-O/EXP-R	TTTGGTATGCGTTAGAACAGTGGTCGTTACAAATTTGGTCAGCTCTTTTTGCGGCAAGAGCAATTTGTAAAAGTGCAAAGAATAATGGAGTTGGTCTCATATTTGAATTCAATTGTGATG	This study
ORF19.251-O/EXP-F	GCGTTGTTATATTGTTGATGAATCGAAGAAAACTAGATTACTAATTGAAATGGTACAAAATAGTTCAACCGTTTCAAGTTTTCAATTATCAGAATTTTCGATCAAGCTTGCCTCGTCCCC	This study
ORF19.251-O/EXP-R	CAAAAGGATGTAAAGCTTCAACAACGAAAACACCAGTCTTTTTACCGTCACTGTAAAAAGTTTCGTTATAAGAAGTAAGAGCGAGTAAAACTTTGACCATATTTGAATTCAATTGTGATG	This study
AQY1-O/EXP-F	TATACTTTCTAGAAATAAGAAAAGGGTTACCCTATCACTATTCTTCTCTTTGGTTAGTTACCAAAACCAAATTCTACTTCTTGGACAACTTCTTTTCCCT ATCAAGCTTGCCTCGTCCCC	This study
AQY1-O/EXP-R	GCGACACATTGACAGAATCGTCATATTTTGGTTCATAGACTGGTCTTTGAGCCTCAACGTCGTTAGGAGTATTATCAATGCTAGAACTTTCGGCAACCAT ATTTGAATTCAATTGTGATG	This study
ORF19.1862-O/EXP-F	TTGTGGATCCAATACAGCTATTGTAGTGGTACTTACTGAAATGTAATCACTTCGCATTATTCAAGATCTTTTTCTATTGTGGCTGGTTTTTGGTGATTGC ATCAAGCTTGCCTCGTCCCC	This study
ORF19.1862-O/EXP-R	TTATATTTGGCTAACAAATCGGCATCATCGATGAAAATGACAAAATCGTTTTCAGCACCTTTGTAGAATAATTTATGTGGAGAAGTTTTAGCCATATTTGAATTCAATTGTGATG	This study

Primer Name	Primer Sequence	
ORF19.4216-O/EXP-F	AAACAGCCTAGTGATAATAAGTGTGAAGAGTAGTAATTGAAGTTTGGTGTGTCTAGGTGGTGTTTAAGACTGTTTAGTATTTTCGCAAGAATTTTGTTAACT ATCAAGCTTGCCTCGTCCCC	This study
ORF19.4216-O/EXP-R	TTATGGTGTATGTCGAAAAAAAACAAGATTCAAAGAATTTACCTCTACTAGAGAAATTGACTCTATTTATATTGTTTTTCGAGAGGGGGGCAAAGGTTGCCAT ATTTGAATTCAATTGTGATG	This study
CSP37-O/EXP-F	GAATAGTCACAGAGAGAGAGAGAAGGATTGCTATTTTTATTTTTTTGGGGGCATAATCACTTATTATACGTCACCACCACCACTAATGGATATATGATTC ATCAAGCTTGCCTCGTCCCC	This study
CSP37-O/EXP-R	GGTGTTGTTGTCTTGGTAAGATTGGTTGAACATATTGATCATAATAATAAACCCCACCGACAAGGGCAGCAGTACCTAATAAATATTTTCCAGCAGACATATTTGAATTCAATTGTGATG	This study
NAT-OE-R-DET	GAA ACA ACA ACG AAA CCA GC	Nobile *et al.*, 2006
ALS2-F-O/EXP	ACTTCTTCAAATACTAAAACGTTTTCACATAAACTGCAAGCCTCAAATTGATAGTATTCTACAGTATAAATGCTGAACTAACTTATTTTTACAATTACAA ATCAAGCTTGCCTCGTCCCC	This study
ALS2-R-O/EXP	TTGTCCATGTCAACGAATCAAAACTATTGAAAACACCCGTAATAACTTTTGCAGTAGCAACTGATACACAGAGGCTTAGCAACAAAAATTGTAAAAGCAT ATTTGAATTCAATTGTGATG	This study
ALS4-F-O/EXP	TTGATAGTTCTCTCCAGTATAAATGCTGAATTAACTCATTTTTACAATTACAAATTAGTAATTATGCATTTGAAAATGGTGCTTACGTTTGATTTCAATG ATCAAGCTTGCCTCGTCCCC	This study
ALS4-R-O/EXP	TGGTCCAAGTTAACGAATTAAAACTATCGAAAATGCCCGTAATAACCTTTGCCGTAGCAACTGATACACAGAGGCTTAGCAACAAAAATTGTAAAAGCAT ATTTGAATTCAATTGTGATG	This study
ALS1-O/EXP-F	GAATCTGCAATGAAAACGTTAAGACATTGGAATTTTTCATCAAATTTACGATGAATTGCTAATCATCTTTGGAGATATTCGTAGTAAGATCTTCAACCCA ATCAAGCTTGCCTCGTCCCC	This study
ALS1-O/EXP-R	TGGACCAAGTTAATGAATTAAAACTATCAAAAACACCAGTGATTGTCTTTGCACTTGCAATTGACAAATATAGGAATAACAATGTAAATTGTTGAAGCAT ATTTGAATTCAATTGTGATG	This study
WH11-O/EXP-F	CTAAATTTCTTCTTATTTCTACATTTAAGGGAACACCCTATTCAACCTATGGAATTGTGATTTCACCTTGAAATCCTCGGGATCTGCATAAACTAAGTTG ATCAAGCTTGCCTCGTCCCC	This study
WH11-O/EXP-R	TAACCTTATCCTTGAATTGTTCTGGAGTGGATTTTTGAGAATCTGGAGTTAATTTGGATTCGATTTTATCACCAATATCTTTTCTACCTAAGTCGGACAT ATTTGAATTCAATTGTGATG	This study
CSH1-F-O/EXP	ACGGAATGCCATCAACTGTTGTGAGAAACTTGGGACTATAGTATAGAGAACATTCAAGCTTGGTGTTGTTATAGATGATTTGATTAATAATGTAAAAAAT ATCAAGCTTGCCTCGTCCCC	This study
CSH1-R-O/EXP	CGATGTCACCATTAAAACCTCTCCAACTGGATCCCAATCTCATAGTACCGACAGCAACAGTGTTGACCTTCAAACCAGATTTACCTAATCTGGTGACCAT ATTTGAATTCAATTGTGATG	This study
ECM331-F-O/EXP	GGTGGTAGTAATGACAATTTGATAGTATTGTTACTTTTCGTGTTTTCTAACTCATTTATACAACATTCCTTTTGTTGCATTACTTTACTATAGCAACAAT ATCAAGCTTGCCTCGTCCCC	This study
ECM331-R-O/EXP	TGGAAGTTTTAGAGAATGAACATTTGTTTGATGAGTCTGCTGCTGAAACTGATGTCAATAAGGCAGCGACTATTGGTAAAAGAAATGACTTAATTTGCAT ATTTGAATTCAATTGTGATG	This study
TPK1-SAT1-F1	ACTGTCACGCAA GGTACCAAGTTTTAACTAACTA	This study
TPK1-SAT1-R1	AACTTCTCGAGCAGAAACAGTGTTGAATAATATCACCTT	This study
TPK1-SAT1-F2	TAATTAATTTGCCCGCGGAAGAAAGAACAAGAAA	This study
TPK1-SAT1-R2	AGACTTGACAAACGAGCTCTTTAGTATCACCGTA	This study