Diversity of $\text{Ca}^{2+}$-Induced Morphology Revealed by Morphological Phenotyping of $\text{Ca}^{2+}$-Sensitive Mutants of *Saccharomyces cerevisiae*

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Cellular morphology is tightly linked to cellular processes and functions in virtually all eukaryotic organisms. In the yeast *Saccharomyces cerevisiae*, the elliptical shape sequentially changes with cell cycle progression under nutritionally rich conditions (9, 24). Under particular conditions, yeast cells drastically change their morphology, forming the shmoop tip (pear-like cell morphology) under mating conditions (29) and pseudohyphae (filamentous cell morphology) or ascospores (four cells within a cell) under starvation conditions (15, 19).

Since cell morphology changes during the yeast cell cycle, drastic morphological changes can be induced in yeast cells by adding reagents that inhibit cell cycle progression. Large-budded cells accumulate following treatment with hydroxyurea (14), which inhibits ribonucleotide reductase. Treatment of *zds1* (shion different screens) (2, 33) mutant cells with 50 to 300 mM $\text{Ca}^{2+}$ induces $G_2/M$ arrest and the formation of elongated buds with one nucleus at the mother/bud neck, which indicates that the $\text{Ca}^{2+}$-signaling pathway mediates cell cycle control via repression of the *SWE1* transcription in $G_2$ phase (17). Genes that are involved in the Zds1-mediated $\text{Ca}^{2+}$-signaling pathway have been identified (18, 31). However, Zds1-dependent, $\text{Ca}^{2+}$-mediated, cell cycle control may be only a part of the $\text{Ca}^{2+}$-signaling pathway, since many $\text{Ca}^{2+}$-sensitive (cls) mutants have been reported (6, 12, 21). Ohya et al. identified 18 complementation groups (unsaturated) of cls mutants (21), some of which have been implicated in intracellular $\text{Ca}^{2+}$ homeostasis (10, 23). It remains unknown whether the morphological features and cellular functions of these cls mutants are similar to those of the *zds1* mutant.

In the present study, we analyzed 58 cls mutants for morphological changes induced by $\text{Ca}^{2+}$ based on high-dimensional and quantitative morphological information obtained using the CalMorph program (22). Our statistical and multivariate analyses reveal that wild-type (WT) yeast cells undergo various types of morphological change in response to $\text{Ca}^{2+}$. Thirty-one cls mutants were clustered into seven groups based on shared characteristic changes. This is the first demonstration that quantitative comprehensive phenotypic analyses of yeast mutants can be used to assess reagent-induced effects that cannot be discriminated in the WT strain.

MATERIALS AND METHODS

Media and strains. Libraries of the haploid strains BY4741 (MATa) and BY4742 (MATa) with a deletion of each of the 4,845 nonessential genes were purchased from the *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF: http://www.uni-frankfurt.de/fb15/mikro/euroscarf/2000). The medium for growing *S. cerevisiae* was YPD medium that contained 1% (wt/vol) Bacto yeast extract (BD Biosciences), 2% (wt/vol) peptone (WAKO), and 2% (wt/vol) dextrose. YPD pH 5.5 medium was YPD medium that was buffered to pH 5.5 with 50 mM succinate-NaOH. For examination of $\text{Ca}^{2+}$-sensitivity, YPD medium supplemented with 100 mM CaCl$_2$ was used as a $\text{Ca}^{2+}$-rich medium. YPG medium (1% Bacto yeast extract, 2% peptone, 2% [vol/vol] glycerol) was used for examination of the Pet$^+$ phenotype. To assess sensitivities to other divalent cations, YPD medium supplemented with 100 mM MgCl$_2$, 3

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mM ZnCl₂, and 5 mM MnCl₂ was used. Plates that contained YPD medium supplemented with 150 mM KCl were used to assess osmotic pressure sensitivity. Solid media were prepared by adding 2% (wt/vol) agar to the above media.

**Growth and test conditions.** For measurement of the growth rate, each strain was grown in YPD liquid medium in the wells of microtiter plates (round-bottomed 96-well plates with lids; IWAKI) for 24 h at 25°C. The cultures were diluted to 3 × 10⁶ cells/ml in YPD or YPD medium that was supplemented with CaCl₂ to a final concentration of 100 mM and then were incubated at 30°C with agitation. The optical density at 630 nm was measured in an MTP120 microtiter plate reader (Corona Electric).

**Observations of vacuolar acidification.** Log phase yeast cells were incubated in 1 ml of YPD medium that contained 50 mM sodium phosphate buffer (pH 7.5) and a 0.5 mM concentration of freshly prepared quinacrine (Sigma) for 30 min at 25°C. Cells were washed twice with 1 ml of YPD medium and suspended in 50 μl of YPD medium. Accumulation of quinacrine in the vacuoles was observed by fluorescence microscopy (BX60; Olympus) with excitation at 395 nm and emission through an NV filter (Olympus) at a 495-nm maximal transmission.

**Measurement of intracellular calcium.** Intracellular calcium content was measured as described previously (6) with some modifications. Yeast cells growing exponentially (1 × 10⁷ to 1.5 × 10⁷ cells/ml) in YPD pH 5.5 medium were collected, resuspended in YPD pH 5.5 medium that contained 45 mM CaCl₂ (20 μM chloroquine; GE Healthcare), and incubated at 30°C for 6.5 h. Aliquots (0.2 ml) were diluted into 1 ml of ice-cold buffer A (5 mM morpholinoethanesulfonic acid-Tris [pH 6.5] and 10 mM CaCl₂) and filtered rapidly onto a 96-well GF/F Unifilter (Whatman). The filter was washed three times with ice-cold buffer A, dried at 80°C for 1 h in an oven, and resuspended in MicroScint-20 scintillation cocktail (Perkin Elmer), and the radioactivity was measured with a TopCount microplate scintillation counter (Perkin Elmer). To determine the intracellular calcium content, each strain was incubated without 45CaCl₂ under the same conditions as the 45CaCl₂-treated cells. Cultures were collected and suspended in 0.2 ml of 10% trichloroacetic acid. The protein content was measured using a bicinchoninic acid protein assay kit (Pierce).

**Fluorescence staining and microscopy.** Cells (8 × 10⁵ cells) at log phase in YPD medium were collected, washed once in YPD medium with or without 100 mM CaCl₂, and resuspended in 4 ml of the respective medium to a final concentration of 2 × 10⁶ cells/ml. The cells were incubated for 5 h at 30°C, washed once with YPD medium, and fixed in YPD medium that was supplemented with 3.7% formaldehyde and 0.1 M potassium phosphate buffer (pH 6.5). Trifle staining of the yeast cells and image analysis with CalMorph were performed as described previously (23). CalMorph automatically characterizes each yeast cell using 501 morphological parameters. Ten and 5 independent cultures grown under the two different conditions were analyzed for the WT and mutant cells, respectively.

**Assessment of parameters changed by Ca²⁺ treatment.** All the statistical analyses were performed using R (http://www.r-project.org/). To investigate the Ca²⁺ effect on yeast cell morphology, we tested the difference between the 10 high Ca²⁺ and 10 low Ca²⁺ values using the U test for each parameter (16). The number of parameters expected to be detected by chance was estimated by empirical permutation tests (4).

**Data preprocessing.** To generate uniform distributions of each parameter value, we transformed the parameter values to statistic

\[
U^* = T - \frac{m(m + n + 1)}{2}
\]

where \(m\) and \(n\) are the sample sizes of the test samples and controls, respectively. The probability distribution of \(U^*\) is the same as the Mann-Whitney statistic \(U\). However, the larger the sample value, the larger the value of \(U^*\) becomes. \(U^*\) was set to 0 when the distribution of the samples was equal to that of the controls.

**Hierarchical clustering.** Hierarchical clustering was performed using R (http://www.r-project.org/). TreeView, version 1.60, was used to visualize the clustering results (7). Clusters were assessed using the R package pvclust tool (http://www.is.titech.ac.jp/~shimo/prog/pvclust/) at a \(P\) value of \(<0.05\) (27). The following options in pvclust were used: method.hclust = “average”; nboot = 1,000; r = seq(0.5, 1.4, by = 0.1). For dissimilarity, see the “Metrics” section below.

**Metrics.** The morphological dissimilarity metric that we used is a form of angle. Let the morphological vector \(\bar{m}\) equal an ordered set of \(U^*\) for the mutants. For any two mutants \(\bar{a} \in \bar{m}\) and \(\bar{b} \in \bar{m}\), the morphological dissimilarity score can be computed as follows:

\[
\delta(a, b) = \arccos \left( \frac{\bar{a} \cdot \bar{b}}{|\bar{a}| |\bar{b}|} \right) \times 360 \div 2\pi
\]

The parameter dissimilarity metric that we used is a form of Spearman's rank correlation coefficient. Let \(P\) equal the rank order of the primary data for parameter \(P\) in independent culture. For any two parameters \(X \in P\) and \(Y \in P\) observed over a series of \(N\) cultures combined under both conditions, the parameter dissimilarity score can be computed as follows:

\[
\delta(X, Y) = 1 - \frac{6}{N(N - 1)} \sum_{i=1}^{N} (X_i - Y_i)^2
\]

**RESULTS**

**Changes in yeast morphology caused by a high concentration of extracellular Ca²⁺.** To investigate the effect of extra-cellular Ca²⁺ concentration on WT yeast morphology, we compared the cellular morphologies of WT yeasts grown under two culture conditions (low and high concentrations of Ca²⁺). For simplicity, WT cells treated with low and high concentrations of Ca²⁺ are referred to as WT-Ca²⁺ and WT+Ca²⁺, respectively. Samples from 10 independent cultures under each condition were characterized by triple-staining fluorescence microscopy and automated cell imaging, as described previously (22). At least 200 cells were analyzed per culture to quantify 501 morphological parameters. For each parameter, we tested the difference between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16). Table 1 shows the significant morphological differences that were noted between the 10 WT-Ca²⁺ and the 10 WT+Ca²⁺ values using the Mann-Whitney test (16).

We also investigated the profiles of the Ca²⁺-dependent morphological changes. Among the 100 parameters detected, we focused on 59 parameters that represented a mean of the measured cells, since the remainder of the parameters represented coefficients of variations in the measurements. When we applied average-linkage hierarchical clustering with dissimilarity based on the Spearman rank order correlation coefficient, we found tightly clustered parameters (Fig. 1). According to these classified parameters, we illustrate the Ca²⁺-dependent morpho-
| Parameter identifier | Description | $P$ value$^a$ |
|----------------------|-------------|--------------|
| **Parameters with significantly increased values** | | |
| C115_A | Whole-cell axis ratio | 1.083E-05 |
| CCV103_C | Coefficient of variation of C103_C | 1.083E-05 |
| CCV115_A | Coefficient of variation of C115_A | 1.083E-05 |
| CCV12-1_C | Coefficient of variation of C12-1_C | 1.083E-05 |
| D107_A1B | Ratio of D107 to C103 | 1.083E-05 |
| CCV103_A1B | Coefficient of variation of C103_A1B | 2.165E-05 |
| D148_A1B | Relative distance of nuclear brightest point to mother center | 2.165E-05 |
| D154_A | Angle between C1D1-1 and C1C1-2 | 2.165E-05 |
| C115_A1B | Mother axis ratio | 4.330E-05 |
| C115_C | Mother axis ratio | 4.330E-05 |
| D147_A1B | Relative distance of nuclear gravity center to mother center | 4.330E-05 |
| C13_A | Whole-cell fitness for ellipse | 7.578E-05 |
| CCV102_C | Coefficient of variation of C102_C | 7.578E-05 |
| D104_A1B | Distance between nuclear gravity center and mother tip | 7.578E-05 |
| D110_A1B | Distance between nuclear gravity center and middle point of neck | 7.578E-05 |
| D114_A1B | Ratio of D110 to C128 | 7.578E-05 |
| DCV154_A | Coefficient of variation of D154_A | 7.578E-05 |
| C114_C | Bud axis ratio | 1.299E-04 |
| CCV110_A1B | Coefficient of variation of C110_A1B | 1.299E-04 |
| D105_A | Ratio of D102 to C103 | 1.299E-04 |
| D118_A1B | Distance between nuclear gravity center and mother center | 1.299E-04 |
| D129_A1B | Distance between brightest point and mother tip | 1.299E-04 |
| D132_A1B | Mobility of nucleus in mother | 1.299E-04 |
| D139_A1B | Angle between C4-1D1-1 and C4-1C1 | 1.299E-04 |
| DCV147_A1B | Coefficient of variation of D147_A1B | 1.299E-04 |
| CCV106_A1B | Coefficient of variation of C106_A1B | 2.057E-04 |
| CCV112_C | Coefficient of variation of C112_C | 2.057E-04 |
| CCV114_C | Coefficient of variation of C114_C | 2.057E-04 |
| D170_A1B | Angle between C4-1D2-1 and C4-1C1 | 2.057E-04 |
| DCV148_A1B | Coefficient of variation of D148_A1B | 2.057E-04 |
| D132_A1B | Distance between nuclear brightest point and middle point of neck | 4.871E-04 |
| CCV116_C | Coefficient of variation of C116_C | 4.871E-04 |
| D142_A1B | Distance between nuclear brightest point and mother hip | 4.871E-04 |
| D155_A | Angle between C1D2-1 and C1C1-2 | 4.871E-04 |
| CCV111_C | Coefficient of variation of C11-1_C | 7.253E-04 |
| CCV12-1_A1B | Coefficient of variation of C12-1_A1B | 7.253E-04 |
| D126_A1B | Distance between nuclear gravity center and mother hip | 7.253E-04 |
| D129_C | Angle between C4-1D1-1 and C4-1C1 | 7.253E-04 |
| C106_A1B | Bud direction | 1.050E-03 |
| D163_C | Angle between D2-1D2-2 and C1C4-1 | 1.050E-03 |
| D170_C | Angle between C4-1D2-1 and C4-1C1 | 1.050E-03 |
| DCV155_A | Coefficient of variation of D155_A | 1.050E-03 |
| D136_A1B | Distance between nuclear brightest point and mother center | 1.050E-03 |
| C13_A1B | Bud cell fitness for ellipse | 2.089E-03 |
| CCV101_A1B | Coefficient of variation of C101_A1B | 2.089E-03 |
| D162_C | Angle between D1-1D1-2 and C1C4-1 | 2.089E-03 |
| D162_A1B | Distance between nuclear gravity center and mother hip | 2.089E-03 |
| D141_A1B | Coefficient of variation of D141_A1B | 2.089E-03 |
| CCV114_A1B | Coefficient of variation of C114_A1B | 2.089E-03 |
| D114_A1B | Coefficient of variation of C115_A1B | 6.841E-03 |
| D148_A | Relative distance of nuclear brightest point to cell center | 6.841E-03 |
| D172_A1B | Angle between C4-1D4 and C4-1C1 | 6.841E-03 |
| DCV118_A1B | Coefficient of variation of D118_A1B | 6.841E-03 |
| C114_A1B | Bud axis ratio | 6.841E-03 |
| CCV115_A1B | Coefficient of variation of C115_A1B | 6.841E-03 |
| DCV112_C | Coefficient of variation of C112_C | 6.841E-03 |
| D155_A1B | Angle between C1D2-1 and C1C1-2 | 6.841E-03 |

Continued on following page
logical changes based on the features reflected in two or more parameters (Fig. 2). In addition to the global changes observed in all cell cycle stages, stage-specific changes were observed. Under the WT+Ca\textsuperscript{2+} condition, rounded cells with centered nuclei accumulated in all stages. In addition, cells with round nuclei accumulated in the unbudded stage, cells with a straightened budding site and direction with a wide neck and large actin region accumulated in all budded stages and cells with proximity of the actin patch to the neck accumulated in the stage of budded cells with two nuclei (Fig. 2). These results clearly indicate that the morphological differences between WT-Ca\textsuperscript{2+} and WT+Ca\textsuperscript{2+} cells occur at different stages of the cell cycle and reflect various cellular aspects.

**Isolation and characterization of novel calcium-sensitive mutants.** We systematically screened novel cls mutants using a yeast deletion mutant collection that covered all the nonessential genes. Initially, the growth rates of all the nonessential deletion strains of the MATA haploid collection were compared on YPD plates and YPD plates supplemented with 100 mM CaCl\textsubscript{2}. The growth rates of the candidate cls mutants in liquid medium were then determined. We defined the cls mutants as follows (Table 2): mutants showing robust growth in YPD medium and defective growth (optical density of <0.3) in YPD plus 100 mM CaCl\textsubscript{2} medium at the time of full growth in YPD medium (growth type A); mutants showing slow growth even in YPD medium and growth defects in YPD plus 100 mM CaCl\textsubscript{2} medium after 60 h of incubation (growth type B); and mutants having an optical density in culture of less than half that of YPD-grown cells after 20 to ~24 h incubation in YPD plus 100 mM CaCl\textsubscript{2} medium (growth type C). Replacement of the yeast genes by the kanamycin resistance gene cassette was verified by PCR using genomic DNA as a template. The MATA cls strains showed the same phenotypes as the corresponding MATA cls strains. As a result, 58 cls mutants were identified (Table 2). All 10 previously cloned nonessential CLS genes were identified in this screening (23, 28, 30). The cls\textsuperscript{+/} (cdc24) mutant was not among the screening strains because CDC24 is an essential gene (5, 20). Twenty-eight cls mutants were common to the recently isolated 64 mutants that are sensitive to 60 mM CaCl\textsubscript{2} (pH 7.5) (26).

Table 2 also summarizes the basic characteristics of the 58 cls mutants, including divergent cation sensitivity, vacuolar acidification, Pet\textsuperscript{−} phenotype (inability to grow on a nonfermentable carbon source), and intracellular Ca\textsuperscript{2+} content. As for

| Parameter identifier | Description | P value\textsuperscript{a} |
|----------------------|-------------|-----------------|
| CCV128_A1B           | Coefficient of variation of C128_A1B | 1.150E-02 |
| D135_A               | Distance between nuclear brightest point and cell center | 1.150E-02 |
| A7-1_C               | Size of actin region in mother | 1.469E-02 |
| D127_A               | Distance between nuclear brightest point and cell tip | 1.469E-02 |
| D1910-1_C            | Coefficient of variation of D1910-1_C | 1.469E-02 |
| A104_C               | Relative distance of actin patch center from neck in bud | 1.854E-02 |
| CCV103_A             | Coefficient of variation of C103_A | 1.854E-02 |
| D141_C               | Distance between nuclear brightest point in mother and mother hip | 1.854E-02 |
| D147_A               | Relative distance of nuclear gravity center to cell center | 1.854E-02 |
| ACV101_A             | Coefficient of variation of A101_A | 2.323E-02 |
| C109_C               | Neck width | 2.323E-02 |
| D103_C               | Distance between nuclear gravity center in mother and mother tip | 2.323E-02 |
| D1213_C              | Coefficient of variation of D1213_C | 2.323E-02 |
| DCV149_C             | Coefficient of variation of D149_C | 2.323E-02 |
| A101_A1B             | Actin region ratio in whole cell | 2.881E-02 |
| A107_C               | Actin c ratio | 2.881E-02 |
| CCV118_C             | Coefficient of variation of C118_C | 2.881E-02 |
| D125_C               | Distance between nuclear gravity center in mother and mother hip | 2.881E-02 |
| D128_C               | Distance between nuclear brightest point in mother and mother tip | 2.881E-02 |
| D146_C               | Distance between nuclear outline point D8 in bud and bud tip | 2.881E-02 |
| D143_A1B             | Coefficient of variation of D143_A1B | 2.881E-02 |
| C105_A1B             | Neck position | 3.546E-02 |
| C127_A1B             | Thickness difference of cell wall | 3.546E-02 |
| D121_C               | Distance between nuclear gravity center in bud and bud tip | 3.546E-02 |
| DCV137_C             | Coefficient of variation of D137_C | 3.546E-02 |
| ACV7-1_A             | Coefficient of variation of A7-1_A | 4.326E-02 |
| ACV7-1_A1B           | Coefficient of variation of A7-1_A1B | 4.326E-02 |
| C107_C               | Long axis length in bud | 4.326E-02 |
| C109_A1B             | Neck width | 4.326E-02 |
| CCV105_A1B           | Coefficient of variation of C105_A1B | 4.326E-02 |
| CCV109_C             | Coefficient of variation of C109_C | 4.326E-02 |
| D102_A               | Distance between nuclear gravity center and mother tip | 4.326E-02 |
| D154_C               | Angle between C1D1-1 and C1C1-2 | 4.326E-02 |
| D182_A               | Nuclear axis ratio | 4.326E-02 |
| DCV150_C             | Coefficient of variation of D150_C | 4.326E-02 |

\textsuperscript{a} P values were estimated by applying the U test to the samples of 10 versus 10 obtained from independent cultures under conditions of low and high concentrations of Ca\textsuperscript{2+}.
FIG. 1. The mean parameters showing differences in the effects of high and low concentrations of Ca\(^{2+}\) on WT yeast cells. The parameters are listed after hierarchical clustering using the Spearman rank order correlation coefficient as the dissimilarity. Red and green boxes indicate the parameters that show higher and lower values, respectively, under conditions of high concentrations of Ca\(^{2+}\) than under conditions of low concentrations of Ca\(^{2+}\). Magenta and navy diamonds indicate the parameter values of 10 experiments in the presence of high and low concentrations of Ca\(^{2+}\), respectively. The minimum and maximum values of 20 experiments are also indicated. The light-green, light-red, and light-blue boxes in the "related morphology" column indicate parameters that are derived from the cell wall, actin, and DNA staining, respectively. The A, A1B, and C in the stage column indicate unbudded cell, budded cell with single nucleus, and budded cell with two nuclei, respectively.
of the growth phenotypes in YPG medium revealed that these strains are not sensitive to osmotic pressure. Observations that was supplemented with 150 mM KCl, which indicates that cation sensitivity. All of the cls sensitivity, whereas all of the vma levels, respectively, than the WT strain (1.06.

concentration of extracellular Ca$^{2+}$

FIG. 2. Illustration of morphological changes induced by extracellular Ca$^{2+}$ in WT cells. The $+\text{Ca}^{2+}$ and $-\text{Ca}^{2+}$ designs indicate high and low concentrations of Ca$^{2+}$ in the medium, respectively. The grey, blue, and red circles in the yeast cells indicate the cell wall, nucleus, and actin, respectively.

divalent cation sensitivity, 16 strains showed Ca$^{2+}$-specific sensitivity, whereas all of the vma mutants showed broad divalent cation sensitivity. All of the cls mutants grew in YPD medium that was supplemented with 150 mM KCl, which indicates that these strains are not sensitive to osmotic pressure. Observations of the growth phenotypes in YPG medium revealed that 45 cls strains had the Pet$^-$ phenotype. Based on the results of quinacrine staining, 23 cls strains showed no vacuolar acidification, and 8 cls strains exhibited abnormal vacuolar morphology. From measurements of Ca$^{2+}$ content with $^{42}\text{Ca}$ uptake, 22, 12, and 24 strains showed higher (>150% of the WT level), similar, and lower (<70% of the WT level) intracellular Ca$^{2+}$ levels, respectively, than the WT strain (1.06 ± 0.08 nmole calcium/mg of protein).

Morphological changes in cls mutants induced by a high concentration of extracellular Ca$^{2+}$. It has been reported that several yeast mutants change morphology under conditions of high Ca$^{2+}$. For example, a calcium-sensitive cls4 mutant of S. cerevisiae stops dividing in the presence of 100 mM Ca$^{2+}$, producing large, rounded, and un budded cells (20). In addition, the zds1 mutant shows defective growth in rich medium in the presence of 50 to 300 mM Ca$^{2+}$, with the cells forming an elongated bud and most of the elongated cells having a single nucleus at the mother/bud neck, which is characteristic of G$^2$ delay (17). Therefore, we expected that some of the cls mutants would change their morphology in the presence of a high concentration of Ca$^{2+}$. We analyzed the morphological changes of 60 strains, including the WT and zds1 mutant, in addition to the 58 cls mutants from five independent cultures under conditions of low and high concentrations of Ca$^{2+}$ in the medium. We used the Mann-Whitney test (16) at a P value of <0.01 to detect differences between the two conditions for each parameter and counted the number of parameters significantly changed in each mutant (Fig. 3). The morphological changes of zds1 mutant cells were observed in 13 parameters (Fig. 3). Among them, C114_C (bud axis ratio of budded cell with two nuclei) gave the most significant increase. In addition to confirmation of Ca$^{2+}$-induced elongated bud morphology in the zds1 mutant, we describe another 12 phenotypes associated with the zds1 mutant in the presence of high concentrations of Ca$^{2+}$ (Fig. 3). The number of significantly changed parameters detected in at least one strain was 278 out of 501, whereas 128 of these would be expected to be detected by chance (see Materials and Methods). Since the WT showed morphological changes in 11 parameters (Fig. 3), fewer than three of which would be expected to be detected by chance, the number of significantly changed parameters in the cls mutants was 10-fold higher than that detected in the WT strain. This suggests that the intensity and diversity of the effect of extracellular Ca$^{2+}$ on morphological change vary for each strain.

The cls mutants can be functionally classified based on the similarities of morphological changes induced by Ca$^{2+}$, since our quantitative morphological analysis of yeast deletion mutants reveals that similar morphological changes are induced when similar intracellular functions are affected (22). In order to represent the similarities of the morphological changes between mutants, the modified U statistic was used in the data processing (see Materials and Methods). We applied hierarchical clustering analysis to the morphological vectors of cls mutants using the positive angles between two arbitrary vectors as the dissimilarity (Fig. 4). The red and green boxes in Fig. 4 indicate increase and decrease of parameter values by Ca$^{2+}$ treatment, respectively. Several mutants showed similar color patterns, suggesting that they share similar Ca$^{2+}$-induced morphological changes. On the other hand, different color patterns are shown in other strains, suggesting that there is a wide variety of the response pattern in the cls mutants.

To verify that the structure in Fig. 4 is of biological origin and is not an artifact of the clustering procedure, the initial data from the yeast morphological response experiment transformed into the modified U statistic were randomized in three different ways and were clustered using the same procedure (see Fig. S1 in the supplemental material) (7). No similar structure resulted from any of these randomized data sets, indicating that the patterns seen in Fig. 4 depict a biological order in the environmental responses of the strains. Subse-
| No. | Gene name (standard/systematic) | Growth type\(^a\) | Divalent cation sensitivity | Pet phenotype | Quinacrine\(^b\) | Ca\(^{2+}\) content (m mole Ca\(^{2+}\)/mg of protein) | Class\(^c\) |
|-----|--------------------------------|-----------------|----------------------------|--------------|--------------|---------------------------------|-----------|
| 1   | afg3/yet017c                   | C               | Ca                         | –            | Yes          | 1.41 ± 0.03                      | II        |
| 2   | bud325/yer014c-a                | B               | Ca                         | –            | Yes          | 2.78 ± 0.10                      |          |
| 3   | bud325/yer026c                 | B               | Ca                         | –            | Yes          | 0.418 ± 0.025                    |          |
| 4   | cls2/hrb036c                   | A               | Ca                         | +            | Yes          | 1.17 ± 0.03                      |          |
| 5   | ctr1/yrp124w                   | A               | Ca                         | –            | Yes          | 0.462 ± 0.043                    | VII      |
| 6   | cwh3606/c007c                  | B               | Ca                         | –            | No           | 0.461 ± 0.031                    | VII      |
| 7   | fet3/ymr058w                   | A               | Ca                         | –            | Yes          | 1.17 ± 0.06                      | III      |
| 8   | ftr1/yer145c                   | A               | Ca                         | –            | Yes          | 1.03 ± 0.06                      | III      |
| 9   | ghl1/ymr046c                   | B               | Ca                         | +            | Yes          | 1.17 ± 0.03                      |          |
| 10  | gom7/ylr184w                   | B               | Ca                         | +            | Yes          | 0.362 ± 0.008                    |          |
| 11  | nol5/yrp072w                   | B               | Ca                         | –            | No           | 2.03 ± 0.06                      |          |
| 12  | och1/ylg038c                   | B               | Ca                         | –            | Yes          | 11.0 ± 0.5                       |          |
| 13  | pdr13/yer064c                  | C               | Ca                         | +            | Yes          | 1.51 ± 0.06                      | II        |
| 14  | pho83/ylp031c                  | A               | Ca                         | Mn           | –            | Yes                              | IV        |
| 15  | pkr1/ymr033w                   | C               | Ca                         | Mn           | +            | No                               | II        |
| 16  | ps10/ydr136w                   | C               | Ca                         | Mg           | +            | Yes                              |          |
| 17  | pmc1/ylg006w                   | C               | Ca                         | +            | Yes          | 0.193 ± 0.008                    | I         |
| 18  | pro1/yrp300c                   | B               | Ca                         | –            | Yes          | 1.21 ± 0.02                      |          |
| 19  | rcs1/ylg071w                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 20  | rib4/ymr143c                   | B               | Ca                         | Zn           | Mg           | –                                 |          |
| 21  | rpl22/yrp061w                  | C               | Ca                         | +            | Yes          | 1.12 ± 0.21                      |          |
| 22  | sac1/ylr121w                   | C               | Ca                         | +            | Yes          | 1.64 ± 0.09                      | V         |
| 23  | sod1/yer104c                   | A               | Ca                         | +            | Yes          | 1.19 ± 0.05                      | IV        |
| 24  | sw3/ylr104c                    | A               | Ca                         | Mn           | –            | Yes                              | I         |
| 25  | tcf4/ylg081w                   | A               | Ca                         | Mn           | –            | No                               |          |
| 26  | tsf3/ylr016w                   | C               | Ca                         | Mn           | –            | Yes                              | II        |
| 27  | trk1/ylr129c                   | C               | Ca                         | +            | Yes          | 1.66 ± 0.14                      |          |
| 28  | ubp3/yer151c                   | C               | Ca                         | –            | Yes          | 1.26 ± 0.05                      | V         |
| 29  | vma1/ylr115w                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 30  | vma10/yrp039w-b                 | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 31  | vma11/ylp234c                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 32  | vma12/ylr119c                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 33  | vma13/yrp036w                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 34  | vma16/yrp026w                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 35  | vma21/yrp127c                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 36  | vma22/yer055w                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 37  | vma3/ylg027w                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 38  | vma4/ylr032w                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 39  | vma5/ylr089w                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 40  | vma6/ylr144c                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 41  | vma7/ylp029c                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 42  | vma8/ylg031w                   | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 43  | vps11/ylr031w                  | A               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 44  | vps15/yer097w                  | C               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 45  | vps16/ylp045w                  | A               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 46  | vps18/ylp148w                  | A               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 47  | vps33/ylg036c                  | A               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 48  | vps34/ylg040w                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 49  | vps36/ylg017w                  | C               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 50  | vps37/ylp095c                  | A               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 51  | vps43/ylg093c                  | A               | Ca                         | –            | Yes          | 0.978 ± 0.026                    | II        |
| 52  | whi3/ylp093c                   | C               | Ca                         | +            | Yes          | 0.847 ± 0.132                    |          |
| 53  | ykl118w\(^d\)                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 54  | yor331c\(^e\)                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 55  | yor999c\(^f\)                  | B               | Ca                         | Zn           | Mn           | Mg                               | –         |
| 56  | zap1/ylg056c                   | C               | Ca                         | +            | Yes          | 1.18 ± 0.12                      |          |
| 57  | zwo1/ylg085c                   | C               | Ca                         | Mn           | +            | Yes                              | II        |

\(^a\) A, normal growth in YPD medium and complete growth defect in Ca\(^{2+}\)-rich medium; B, slow growth in YPD medium and complete growth defect in Ca\(^{2+}\)-rich medium; C, normal growth in YPD medium and partial growth defect in Ca\(^{2+}\)-rich medium.

\(^b\) Y3, vacuole was stained with quinacrine; No, vacuole was not stained with quinacrine; MD, morphological defects in vacuole.

\(^c\) Mutants were classified as described in the legend of Fig. 4.
sequently, to detect robustly clustered strains, we applied the multiscale bootstrap technique (27). At a \( P \) value of \( >0.95 \), we detected seven classes for a total of 32 strains (Fig. 4). Of the seven classes, three (classes III, VI, and VII) included mutants that are already known to be functionally related. On the other hand, when we employed the log-transformed average method (which is the standard preprocessing step) instead of the modified \( U \) statistic, fewer classes consisting of fewer mutants were detected (see Fig. S2 in the supplemental material). In addition, \( vma \) mutants were divided into at least two classes. These results indicate that the modified \( U \) statistic is effective in identifying the class that is consistent with the functionally related mutants.

In order to understand the properties of each class of mutant, the characteristic common morphological changes of each class should be described. We detected the parameters whose values were coherently changed in the same direction in each class at a \( P \) value of \( <0.01 \), and these are summarized in Fig. 5 (see Materials and Methods). The red and green boxes in each class indicate parameters where values were significantly and coherently increased and decreased, respectively, by \( Ca^{2+} \) treatment. The patterns of the colored boxes were different among classes, suggesting that the patterns of morphological changes common in each class are unique to the class (Fig. 5).

When the parameters that represented the coefficient of variance were discarded from all the detected parameters, the numbers of parameters were 37, 105, 28, 28, 28, 64, and 126 in classes I to VII, respectively. As examples, class I mutants contained a large nucleus and showed accumulation of small buds during growth in the \( Ca^{2+} \)-rich medium (see Fig. S3a in the supplemental material). When modified \( U \) values of \( D174\_C \) (maximal distance between nuclear gravity center and nuclear outline in bud) and \( C123 \) (small bud ratio to budded cells) are displayed on a two-dimensional scatter plot, class I mutants are plotted near to each other and are coherently far from coordinate origin (Fig. 6A). Similarly, the cell sizes of class III mutants were considered small because the long-axis lengths of the whole cells were short, and cell roundness was increased (Fig. 6B; see Fig. S3c in the supplemental material), in contrast to the large cell sizes of mutants of classes II, VI, and VII (Fig. 6C; see Fig. S3b, f, and g in the supplemental material). In classes II, VI, and VII, actin delocalization was also observed (Fig. 6C; see Fig. S3b, f, and g in the supplemental material). As shown in Fig. 6D, the bud axis ratio of budded cells gave the most significant increase in the \( zds1 \) mutant. These results suggest that the morphological changes induced by \( Ca^{2+} \) differ among classes and that mutants that do not belong to any class show different morphologies from those of all the other mutants. Therefore, we conclude that \( cls \) mutants show various morphological changes when grown in a high concentration of \( Ca^{2+} \).

**DISCUSSION**

With regard to the high-dimensional and quantitative phenotypic traits, we show that a high concentration of extracellular \( Ca^{2+} \) induces a wide variety of morphological changes in \( cls \) mutants. The quantitative data such as morphological parameters include a dispersion which is the variation of values.
FIG. 4. Cluster analysis of the cls mutants based on the similarities of the morphological changes. Dissimilarity indicates a positive angle (0 to 180°) between the vectors of 501 dimensions (see Materials and Methods). Blue values indicate the AU $P$ value (calculated using the multiscale bootstrap technique) in the dendrogram (27). The orange rectangles indicate robustly clustered mutant classes at a $P$ value of $>0.95$. The red and green boxes indicate modified $U$ statistics that reflect morphological differences from untreated cells. Positive and negative values of the modified $U$ statistic are depicted in red and green, respectively. Class indicates a mutant group that shows similar Ca$^{2+}$-induced morphological changes.
caused by experimental errors. This phenotypic study means that we can input not only the central value (e.g., mean and median) but also the dispersion (e.g., standard deviation and quartile deviation) to the high-dimensional cluster analysis using the data obtained from replicated experiments. Qualitative or categorical phenotypes have been used for classification of mutants without consideration of the dispersion of the data. Recently, in a gene expression profiling study, classes that

FIG. 5. Coherently changed parameters in each class. Dissimilarity indicates a positive angle (0 to 180°) between the vectors of 501 dimensions (see Materials and Methods). Blue values indicate AU the P value (calculated using the multiscale bootstrap technique) in the dendrogram (27). The orange rectangles indicate robustly clustered mutant classes at a P value of >0.95 in Fig. 4. The red and green boxes indicate significantly increasing and decreasing parameters at a significance level of 0.01, respectively.

FIG. 6. Two-dimensional plots of coherently changed parameters in each class. Color areas of yellow, pink, cyan, green, purple, orange, and blue indicate distribution area of parameters in mutants of class I, II, III, IV, V, VI, and VII, respectively. Gray circles and a light green diamond indicate U values of parameters in nonclassified mutants and the zds1 mutant, respectively. Positive and negative values in each axis indicate increase and decrease of parameter values by Ca2+ treatment, respectively. An absolute value of U equal to or higher than 12.5 and 10.5 indicates significant change in each parameter of each mutant by Ca2+ treatment at P values of <0.01 and 0.05 that correspond to a P value of the U test, respectively. Cell cycle stage (A, A1B, and C) is described in the legend of Fig. 1. (A) D174_C (maximal distance between nuclear gravity center and nuclear outline in bud) and C123 (small bud ratio to budded cells). Parameter value of class I mutants (yellow diamonds) coherently increased by Ca2+ treatment (P < 0.01) (see Fig. S3 in the supplemental material). (B) C115_A (whole-cell axis ratio) and C103_A (long-axis length in whole cell). Parameter value of class III mutants (cyan diamonds) coherently decreased in these parameters by Ca2+ treatment at a significance level of 0.01. (C) A112 (cells without localized actin paths in mother) and C101_A1B (whole cell size). Parameter values of the mutants of classes II, VI, and VII (pink, orange, and blue squares, respectively) coherently decreased in A112 and increased in C101_A1B by Ca2+ treatment at a significance level of 0.01. (D) Bud axis ratio in budded cells with one nucleus (C114_A1B) and that in budded cells with two nuclei (C114_C). The C114_C value was significantly increased by Ca2+ treatment in the zds1 mutant (a light green diamond) at a significance level of 0.01 with a U test, whereas it was coherently decreased in class I, II, III, IV, and VII mutants (yellow, pink, cyan, green, orange, and blue squares, respectively). Additionally, the C114_A1B value was coherently decreased in all classes except class V (purple squares) at a P value of <0.01.
consisted of genes with similar expression patterns were estimated by probability (3, 27). In the present study, more accurate and detailed phenotypic analyses were performed to identify classes of mutants with similar phenotypes based on probability. The method that we used is applicable to any quantitative phenotype (even a categorical phenotype) that is obtained from replicated experiments. Therefore, this method represents a powerful method for large-scale phenotypic analysis.

Identification of phenotypically similar classes. Replication of the experiment is important when phenotypes obtained from CalMorph are used for phenotypic analysis. Phenotypic analysis using CalMorph was previously performed by Ohya and coworkers (22). However, they analyzed the data obtained from a single experiment, which was not replicated. Therefore, the data included experimental errors; this makes it difficult to perform detailed analyses, such as the identification of mutants that have similar phenotypes. Using the data set obtained from replicated experiments with the 58 cls mutants, we detected and characterized classes of mutants that showed similar phenotypes at a P value of >0.95, and we detected coherently changed parameters in each class at a P value of <0.01.

The modified test statistic derived from transformation of the data from the replicated experiments enabled us to use parameters with values that showed different distributions for the clustering and to summarize the morphological similarities. Initially, we calculated the U statistic obtained from replicated experiments under the two conditions. Then, the U statistic was modified to reflect the direction of change and applied to the cluster analysis. Since the calculation process is based on the rank-order method, all of the parameter values were transformed to the same distribution irrespective of their original distributions. Therefore, this preprocessing method is applicable to any quantitative phenotypes that show different distributions.

Better clustering results can be obtained using the modified U statistic rather than the log-transformed average. In the analysis of expression data from microarrays, the standard preprocessing involves log transformation of the expression values, i.e., the Cy5/Cy3 fluorescence ratios (7), and the expression values are averaged (11). When the morphological data were applied to the cluster analysis after this standard preprocessing step, few classes that contained functionally related mutants were identified (see Fig. S2 in the supplemental material). On the other hand, when the cluster analysis was performed after preprocessing with the modified U statistic, several classes of functionally related mutants, such as iron ion transporter (class III), class C VPS (class VI), and VMA (class VII), were identified (Fig. 4). These results suggest that the modified U statistic is effective in the classification of functionally related mutants.

Cluster analysis application. Having classified and characterized the cls mutants based on morphological changes, we considered five potential problems: (i) the sample size for the clustering, (ii) lack of unity in the data types of the parameters, (iii) the selection of a dissimilarity metric, (iv) validation of the clustering results, and (v) extraction of characteristic phenotypes. First, it is known that a clustering produced with a sample size of less than 50 (which is typical of a microarray study) is generally not reproducible (8). In the present study, we used 60 samples in the clustering of the mutants.

Second, the data type is restricted to only one type when the cluster analysis is applied (11), although the parameter outputs from CalMorph have data types of length, number of cell, coefficient of variation, etc. To overcome this problem, we made the data type uniform by transforming the parameter values to the modified U statistics.

Third, some dissimilarity metrics, such as Euclidean distance and the correlation coefficient, were expected to group several mutants as “weak-phenotype mutants”, since some mutants were weakly affected by Ca²⁺ (Fig. 3). Therefore, we needed to extract the values that represented the nature of Ca²⁺-induced morphological change from the morphological vectors of the mutants. The magnitude and direction of a morphological vector correspond to the affected intensity and the nature of the morphological change, respectively (see Materials and Methods section). We accomplished the comparison of the nature of morphological change using angles as the dissimilarity.

Fourth, unsupervised classification of high-dimensional data should be validated using resampling-based procedures (1). The validation methods for the clustering results based on the resampling procedures have been reported in the methodology for microarray data analysis (3, 27). The classes that consisted of robustly clustered mutants were identified using the approximately unbiased (AU) P value, which was calculated by multiscale bootstrap resampling (27). We applied this validation method to our clustering data, thereby confirming the results.

Fifth and last, in the past, there have been no suitable methods for the extraction of the characteristic phenotypes of individual classes. To select the characteristic parameters, we estimated the probability of the coherency of changed parameters among the mutants in the identified classes. We discovered that the distribution of the sum of the modified U statistics in each class followed the theoretical distribution of the U statistic. As a result, at a P value of <0.01, many parameters that were coherently changed by Ca²⁺ were detected in each class (Fig. 5).

Morphological changes in cls mutants caused by a high concentration of extracellular Ca²⁺. A high concentration of extracellular Ca²⁺ induced various morphological changes in the WT yeast strain (Fig. 2). In the WT, 13% of the parameters (65/501) were detected at a P value of <0.01 between the two conditions for 10 treated samples (Table 1). This is a strikingly high number of affected parameters, since deletion mutants with the same or more levels of morphological alteration (33/254) numbered 92, which represents <2% of the 4,718 nonessential mutants (22). In addition, the effects of a high concentration of extracellular Ca²⁺ on morphological properties were extremely variable, with changes in cell shape, position of the nucleus, location of actin patches, angle of budding, and wideness of neck.

It should be noted that the Ca²⁺ responses of the cls mutants varied more than those of the WT. The number of significantly changed parameters for all the cls mutants for the two conditions (Fig. 3) was at least 10-fold higher than that of the WT, which indicates that a greater variety of morphological changes occurs in the cls mutants. In other words, the use of a comprehensive set of cls mutants enabled the discovery of a higher number of different responses to Ca²⁺.
Some of the cls mutants were robustly clustered by the nature of morphological change induced by Ca\(^{2+}\). In class I, which included the WT, the nuclei of the mutants were larger (Fig. 6A and see Fig. S3a in the supplemental material). The mutants of class VII were vma mutants, which are known to be functionally related. The common phenotypic change to larger cells in this class was consistent with the previously reported phenotype of vma mutants (34). In addition, in this class, decreased actin localization was observed (Fig. 6C and see Fig. S3f in the supplemental material). Actin delocalization was also observed in classes II, VI, and VII, whereas no actin parameters were detected in the WT for the two conditions for five treated samples (P < 0.01). Class VI mutants comprised class C vps mutants, which are also known to be functionally related (25). Class III mutants, which appeared as small cells when grown in a high concentration of Ca\(^{2+}\), are iron ion transporter mutants (13). None of the detected classes included the cls2cag2 mutant, which functions in releasing Ca\(^{2+}\) from the lumen of the endoplasmic reticulum (28). These results suggest that the various intensities and patterns of Ca\(^{2+}\)-induced morphological changes in cls mutants depend on the gene functions.

The observation of an elongated bud under high Ca\(^{2+}\) conditions in the zds1 mutant cell (17) is one of the morphological changes reportedly induced by Ca\(^{2+}\). We confirmed that the zds1 mutant formed elongated buds, since the parameter of the morphological phenotypes of mutants, given that our statistical facilitation of further phenotypic analysis of microarray data. For example, if the expression data on cls2 transporter mutants (13). None of the detected classes in microarray data. For example, if the expression data on cls2 transporter mutants (13). None of the detected classes in microarray data. For example, if the expression data on cls2 transporter mutants (13). None of the detected classes in microarray data. For example, if the expression data on cls2 transporter mutants (13). None of the detected classes in microarray data. For example, if the expression data on cls2 transporter mutants (13). None of the detected classes in microarray data. 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