Function of a Plant Stress-Induced Gene, HVA22. Synthetic Enhancement Screen with Its Yeast Homolog Reveals Its Role in Vesicular Traffic

Alex Brands and Tuan-hua David Ho*

Department of Biology, Washington University, St. Louis, Missouri 63130

Expression of the barley (Hordeum vulgare) HVA22 gene is induced by environmental stresses, such as dehydration, salinity, and extreme temperatures, and by a plant stress hormone, abscisic acid. Genes sharing high level of sequence similarities with HVA22 exist in diverse eukaryotic organisms, including animals, plants, and fungi, but not in any prokaryotic organisms. The yeast (Saccharomyces cerevisiae) HVA22 homolog, Yop1p, has been shown to interact with the GTPase-interacting protein, Yip1p. Deletion of YOP1 led to only a modest reduction of the stationary phase titer at 37°C. A synthetic enhancement mutant screen was performed in the yop1 deletion background to identify genes interacting with YOP1. The open reading frame YOR165W (renamed SEY1 for synthetic enhancement of YOP1) was identified as a YOP1-dependent complementation gene. The yeast SEY1 is a homolog of the Arabidopsis RHD3 gene whose mutations cause the accumulation of transport vesicles near the tips of defective root hairs. The yeast double mutant of yop1 and sey1 is defective in vesicular traffic as evidenced by the accumulation of transport vesicles and the decrease in invertase secretion. Based on these observations, we suggest that Yop1p/HVA22 regulates vesicular traffic in stressed cells either to facilitate membrane turnover, or to decrease unnecessary secretion.

Sessile organisms such as plants and fungi have developed sophisticated responses to environmental stresses that allow them to tolerate adverse conditions (Hohmann and Willem, 1997; Hoekstra et al., 2001). One approach to understanding these responses is through the identification of genes that are up-regulated during abiotic stress, followed by functional analyses of the corresponding gene products. In plants, stress stimulates the production of the phytohormone abscisic acid (ABA) (Zeevaart and Creelman, 1988; Bray, 2002), which induces the expression of a variety of genes (Chandler and Robertson, 1994; Bray, 2002). Furthermore, elevated levels of ABA are correlated with late embryogenesis and the onset and maintenance of seed dormancy (Zeevaart and Creelman, 1988). Because the dehydration of the seed during late embryogenesis is a normal part of the developmental program, these tissues provide a valuable resource for the identification of genes that are involved in desiccation tolerance.

In cereals, a metabolically active tissue, the aleurone layer, surrounds the starchy endosperm of the seed. Upon germination, the embryo produces the phytohormone GA, which induces the production of hydrolytic enzymes by the aleurone tissue. These enzymes are secreted into the endosperm, where they liberate sugars and amino acids for the growing embryo. ABA blocks the production of these enzymes at the transcriptional level (Lovegrove and Hooley, 2000). The gene HVA22 was originally identified as a transcript that accumulates in barley (Hordeum vulgare) aleurone tissue upon treatment with ABA, and was later found to be induced in vegetative tissues exposed to ABA, drought, or cold stress (Shen et al., 1993; Shen et al., 2001). Through RNA-blot analysis and study of promoter structure and activity, the regulation of HVA22 expression has been well characterized (Shen et al., 1996, 2001). However, the role of the HVA22 protein in stress response is not yet understood. Database searches reveal homologs of HVA22 in diverse eukaryotic organisms, including other plants, animals, fungi, and protists (Shen et al., 2001). Arabidopsis has at least five homologs of HVA22, and analysis of their expression patterns shows that they are differentially regulated by hormonal and developmental signals (Chen et al., 2002). It has also been shown that expression of the yeast (Saccharomyces cerevisiae) homolog can be induced by salt stress (Shen et al., 2001).

The yeast homolog of HVA22 was named YIP2 (Ypt-interacting protein) based on a physical interaction with the rab GTPase Ypt1p (http://genome-www4.stanford.edu/cgi-bin/SGD/locus.pl?locus=YIP2;Yang et al., 1998). The Rab GTPases are present in all eukaryotes, and have been established as important players in various aspects of vesicular transport, with their function dependent on coordinated interaction with upstream regulators and downstream effectors. These small proteins cycle between...
GTP- and GDP-bound forms, and this cycling is controlled by the upstream regulators. The GTP-bound form acts on downstream effectors, recruiting them to the site of action (Segev, 2001a).

More recently, YIP2 has been named YOP1 (YIP one partner) based on a physical interaction with Yip1p. Two-hybrid assays, glutathione S-transferase pull downs, and subcellular localization experiments all indicate that Yop1p and Yip1p interact in vivo (Calero et al., 2001). YIP1 encodes an essential membrane-bound protein that interacts with the rab GTPases Ypt1p and Ypt31p, perhaps recruiting them to the Golgi membrane (Yang et al., 1998).

The presence of a homolog of HVA22 in yeast provides the opportunity to study this gene in a versatile model organism. Because deletion of the YOP1 coding sequence results in only a mild phenotype, a synthetic enhancement screen was performed to identify genetic interactions that may provide some insight into the function of Yop1p. Because of the convenient features of yeast genetics, we believe that studying the action of Yop1p will enhance our understanding of the function of HVA22. We report here the results of that screen, characterization of the isolated mutants, and discuss the potential function of the YOP1 and HVA22 proteins.

RESULTS

Barley HVA22, Yeast Yop1p, Human (Homo sapiens) Dp1, and Arabidopsis AtHVA22d Share Sequence Homology and Similar Hydropathy Profiles

Homologs of HVA22 are present in diverse eukaryotic organisms (Fig. 1; Calero et al., 2001; Shen et al., 2001). The region of highest homology is a short hydrophilic loop flanked by two hydrophobic stretches. The C-terminal region shows the highest degree of variability between species, although it is hydrophilic in all cases examined (Fig. 1B). The yeast and human homologs contain a 40- to 48-amino acid N-terminal region that is not present in HVA22 and AtHVA22d.

yor1Δ Has a Mild Temperature-Sensitive Growth Defect

To begin our investigation of YOP1, the coding sequence was replaced by a kanamycin resistance cassette by homologous recombination. The resulting yor1Δ strain, ABY16, was viable under various growth conditions. (See Table I for yeast strains used in this study.) No difference in growth between wild type and the yor1Δ mutant was observed at room temperature under salt stress up to 1.5 M NaCl (data not shown). When grown at room temperature in yeast peptone dextrose (YPD) liquid media, there is no detectable difference in A₆₀₀ between wild type and the yor1Δ mutant (Fig. 2A). However, when cultured at 37°C in YPD, the yor1Δ mutant reached an A₆₀₀ of approximately 75% that of the wild-type strain (Fig. 2B). Viable cell counts were determined by plating dilutions of these cultures. The ABY16 cultures were found to have approximately 93% the cell count of the wild-type cultures. The difference in cell count was the same whether the cultures were grown in separate flasks or in competition (data not shown). The same phenotype was observed in strain ABY14, in which the YOP1 coding sequence was replaced by an HIS3 cassette (data not shown).

yor1Δ Shows a Synthetic Enhancement Defect with sey1

Because the yor1Δ mutant did not show a severe phenotype, a synthetic enhancement screen was performed in a yop1Δ background to identify genetic interactions with YOP1. Thirty-nine non-sectoring (sect−) mutants were isolated, subjected to complementation group analysis, and tested for YOP1 and URA3 dependence. A single YOP1-dependent complementation group, consisting of four individually isolated mutants, was identified.

The mutant ABY51 was transformed with a genomic library, and sectoring colonies recovered. Plasmids from these colonies were isolated and characterized by end sequencing of the genomic inserts. As expected, multiple YOP1 genomic clones were recovered. A 16-kb clone containing eight open reading frames (ORFs) from chromosome 15 was recov-
ered several times. In addition, an overlapping 12-kb genomic clone containing four ORFs was recovered several times. Subcloning fragments of this clone and testing for sect+ led to the identification of ORF YOR165W as the complementing gene (Fig. 3). We named this ORF SEY1 (synthetic enhancement with YOP1). SEY1 is a homolog of the Arabidopsis gene RHD3 (Root Hair Defective 3). Both of these proteins contain the two predicted GTP-binding motifs GXXXXGKS and DXXG near the N terminus (Wang et al., 1997; Fig. 4), although neither has been demonstrated to bind GTP. An LEU plasmid containing a YOP1 or SEY1 genomic clone restored sectoring in the synthetic enhancement mutant, but an empty LEU plasmid did not (Fig. 3).

The N-Terminal Region of Yop1p Is Not Required for Its Function

It has been demonstrated that the N-terminal 17 amino acids of Yop1p are necessary and sufficient for its physical interaction with Yip1p, as determined by the two-hybrid assay (Calero et al., 2001). We wanted to determine if this interaction is necessary for the activity of Yop1p. To test this, a truncated yop1 mutant lacking coding sequence for amino acids 2 through 17 was constructed in a LEU vector. This was transformed into the synthetic enhancement mutant ABY51. Transformants were streaked onto rich media and scored for sectoring. After 1 week of growth, transformants carrying the truncated yop1 showed sectoring (Fig. 3D), whereas those carrying the empty vector did not (Fig. 3A), indicating that the N-terminal region of Yop1p is not necessary for its function.

Characterization of sey1 Alleles

The sey1 alleles were cloned from three of the mutants by gap repair, and the fourth allele was recovered from two independent PCR amplifications. Molecular lesions were identified by sequencing (Fig. 4). Three of the alleles (sey1-1, sey1-2, and sey1-3) result from 1-bp mutations that give rise to amino acid changes: Ser-41-Leu, Gly-106-Asp, and Thr-186-Ile, respectively. All three of these amino acids are conserved between SEY1 and RHD3. The sey1-1 mutation is near the first GTP-binding motif, whereas the sey1-2 mutation disrupts the second GTP-binding motif. Two of the alleles are conditional. The sey1-3 mutant is sect− on YPD, but sectoring on YPD supplemented with 25 mM CaCl2. The sey1-4 mutant contains a G to A base pair change, resulting in a stop codon in place of the Trp-273 codon. Despite this severe truncation resulting in the loss of more than 60% of the predicted protein, this mutant is sectoring at 37°C, but sect− at room temperature (data not shown).

An sey1Δ mutant (BY2421) was obtained from Research Genetics (Huntsville, AL), and its growth was...
compared with its parent wild-type strain (BY4741). When grown at room temperature or 37°C in YPD liquid media, there is no detectable difference in A₆₀₀ between wild type and the sey1Δ/H9004 mutant (Fig. 5).

A yop1Δ/sey1 Double Mutant Is Impaired in Vesicular Transport

YOP1 was previously named YIP2 (Ypt1p interacting protein 2) based on a two-hybrid interaction with the rab GTPase Ypt1p (http://genome-www4.stanford.edu/cgi-bin/SGD/locus.pl?locus=YIP2; Yang et al., 1998). This entry in the database provided the first indication that Yop1p is involved in vesicular transport. In addition, a BLAST search performed with the Sey1p amino acid sequence against the yeast genome shows weak sequence similarity to Uso1p (BLAST score 117, 23% identity, 40% similar), a coiled coil protein believed to be a vesicle tethering factor and required for vesicular transport (Nakajima et al., 1991; Barlowe, 1997), although Sey1p does not appear to have a coiled coil region (data not shown). RHD3, an Arabidopsis homolog of SEY1, had previously been identified in a screen for root hair abnormalities. Close examination of this mutant shows an accumulation of transport vesicles in the tip of expanding root hairs, indicating a problem with vesicular transport (Galway et al., 1997). These published results, taken together with the genetic interaction of SEY1 and YOP1 reported here, also indicate that YOP1 is involved in vesicular transport. To examine the possibility that the yop1Δ/sey1 synthetic enhancement mutants have a defect in vesicular transport, transmission electron microscopy (EM) was used to examine the internal morphology of the mutants.

The yop1Δ/sey1-4 mutant was used to recover white colonies at 37°C, which were grown in liquid media at 37°C, then shifted to room temperature for 3 h. Transmission EM on these cells reveals an accumulation of vesicles and occasional ring-shaped structures known as Berkeley bodies (Novick et al., 1980), Figure 3.

Figure 2. The Yop1Δ mutant shows a temperature-sensitive growth phenotype. Liquid YPD media was inoculated with wild type or yop1Δ (deletion mutant) and shaken at 25°C or 37°C. The density of the cultures was quantified by measuring A₆₀₀. Values shown are averages ± se, n = 3. A, At 25°C, there is no significant difference between wild type (solid line) and the yop1Δ mutant (dashed line). B, At 37°C, the A₆₀₀ of the mutant cultures are about 75% that of wild type after 55 h.

Figure 3. sey1 synthetically enhances yop1Δ. A synthetic enhancement mutant was transformed with pRS315 containing no insert, a YOP1 genomic clone, a SEY1 genomic clone, or an N-terminal deletion mutant of YOP1 and streaked out on rich media. After 1 week, colonies were examined for sectoring.
which are not seen in wild-type, yop1Δ, or sey1Δ cells (Fig. 6).

**The yop1Δ/sey1 Mutant Is Defective in the Secretory Process**

To determine if the accumulation of vesicles correlates with a secretion defect, secreted and total invertase activities of the double mutant were assayed and compared with those of the wild type. Cells were grown to log phase in media containing 5% (w/v) Glc. The yop1Δ/sey1-4 mutant was grown at 37°C, then shifted to room temperature for 3 h. At log phase, invertase expression was derepressed by transferring the cells to media containing 0.05% (w/v) Glc. After 3 h, total and external invertase activity was measured. The mutant shows inefficient secretion of invertase, with only 50% of its total invertase secreted, as compared with more than 80% for wild type (Fig. 7A). Transformation of the mutant with either a YOP1 or YOR165W genomic clone rescued the secretion defect (Fig. 7B).

**Yop1p Interacts with Itself, But Not with Sey1p, in a Two-Hybrid Assay**

Synthetic enhancement indicates a functional relationship between two gene products. In some cases, the gene products physically interact (Rose, 1995). We wanted to test the possibility of a physical interaction between Yop1p and Sey1p. To do this, both the YOP1 cDNA and SEY1 coding sequence were cloned in frame into both the pBD-GAL4 and pAD-GAL4 two-hybrid vectors. Yeast strain YRG-2 (Stratagene) was transformed with one binding domain fusion and one activation domain fusion. Transformants were streaked on to plates lacking His, Leu, and Trp.
After 5 d, growth was monitored. Neither combination of YOP1 and SEY1 fusion constructs allowed for growth, revealing no interaction between the fusion proteins (data not shown). To investigate the possibility that Yop1p or Sey1p form a homodimer, YRG-2 was transformed with both YOP1 fusion constructs or with both SEY1 fusion constructs. Transformants carrying both SEY1 fusion constructs showed no growth (data not shown). Transformants carrying both YOP1 fusion constructs showed growth comparable with the positive control, whereas transformants carrying one fusion construct and one empty vector did not grow (Fig. 8A). Interestingly, when the fusion constructs were made using the YOP1 genomic sequence, which contains one intron, no interaction could be detected (data not shown). Sequence analysis shows that the predicted Yop1p protein is Leu rich and contains a potential Leu zipper (Fig. 8B), a motif characterized by a Leu, Ile, or Val every seventh residue, and allows for dimer formation.

DISCUSSION

Homologs of the plant stress-induced gene, HVA22, are present in diverse eukaryotic organisms. We have shown previously that expression of the yeast HVA22 homolog, YOP1, is also induced by NaCl treatment (Shen et al., 2001) Taking advantage of the versatile genetic features of yeast, we have studied the yeast YOP1 gene as a part of the effort in elucidating the function of HVA22 in stress response. Because a yop1Δ mutant does not have a severe phenotype, a synthetic enhancement screen in a yop1Δ

Figure 7. The synthetic enhancement mutant has a secretion defect that can be rescued by YOP1 or SEY1. A, Wild type or yop1Δ/sey1-4 mutant yeast were grown in yeast peptone (YP) supplemented with 5% (w/v) Glc at 37°C. During log phase, the cultures were washed and resuspended in YP media supplemented with 0.05% (w/v) Glc at 25°C. After 3 h, cells were collected and resuspended in 10 mM NaN3 and processed as in A. Values shown are averages ± se, n = 3. B, The yop1Δ/sey1-4 mutant was transformed with pRS315 containing a YOP1 genomic clone, no insert, or an SEY1 genomic clone. Cultures were grown in synthetic complete-Leu media supplemented with 5% (w/v) Glc at 37°C. During log phase, the cultures were washed and resuspended in synthetic complete-Leu media supplemented with 0.05% (w/v) Glc at 25°C. After 2.5 h, cells were collected and resuspended in 10 mM NaN3 and processed as in A. Values shown are averages ± se, n = 3.
background was performed to identify genetic interactions with \textit{YOP1}. From this screen, a single complementation group, composed of four alleles of \textit{YOR165W}, was identified. We have named this gene \textit{SEY1} (synthetic enhancement with \textit{YOP1}). It is homologous to the Arabidopsis gene \textit{RHD3}, which encodes a protein containing two GTP-binding motifs. These motifs are present in Sey1p as well (Fig. 4; Wang et al., 1997). The interactions between \textit{YOP1}, \textit{SEY1}, and several other yeast genes involved in vesicle traffic are summarized in Figure 9.

A synthetic enhancement interaction can represent several kinds of relationships between gene products. One possibility is simple redundancy, where the two proteins perform the same biochemical function (Rose, 1995). Yop1p and Sey1p do not show any kind of sequence similarity, so it is unlikely that they have the same biochemical activity. Another possible relationship is that of functioning in parallel pathways that have a common endpoint. For example, a Leu auxotroph, such as a \textit{leu2} mutant, will grow normally on media that contains Leu. However, in a genetic background in which a gene required for sensing or transport of extracellular Leu is disrupted, the \textit{LEU2} gene is essential (Nigavekar and Cannon, 2002). A third possibility is that the two gene products physically interact in a complex. Elimination of one of those products may not be enough to disrupt the complex, but elimination or mutation of two components will (Rose, 1995). Two-hybrid analyses do not show an interaction between Yop1p and Sey1p. Furthermore, there is no report of Sey1p physically interacting with any other protein. In contrast, Yop1p has been reported to physically interact with several proteins, including Ypt1p, Yip1p, and Yif1p, as summarized in Figure 9 (Ito et al., 2000, 2001a, 2001b). A synthetic enhancement interaction may also repre-

**Figure 8.** Yop1p interacts with itself in a two-hybrid assay. A, Yeast host strain YRG-2 transformants carrying: i, positive control plasmids pADWT and pBDWT; ii, GAL4AD-YOP1 and GAL4BD; iii, GAL4AD and GAL4BD-YOP1; or iv, GAL4AD-YOP1 and GAL4BD-YOP1 were streaked out on synthetic media lacking Leu, Trp, and His and grown at 30°C for 5 d. B, The amino acid sequence of Yop1p showing a potential Leu zipper (highlighted).

**Figure 9.** Summary of reported interactions involving \textit{YOP1} and \textit{SEY1}. Interactions involving \textit{YOP1} and \textit{SEY1} are shown schematically and are listed as follows: 1 and 2, two-hybrid interaction (Yang et al., 1998); 3, two-hybrid interaction and glutathione S-transferase pull down (Calero et al., 2001); 4, synthetic enhancement (this study); 5, synthetic enhancement (Sapperstein et al., 1996); 6 through 9, two-hybrid interaction (Ito et al., 2001a); 10, sequence similarity (this study).
sent two steps of a single pathway. In this case, disruption of one protein may reduce the efficiency of a single step, but not enough to result in a severe phenotype. However, if two steps in the pathway are disrupted, the overall flux of the pathway may be greatly reduced (Rose, 1995).

We propose that Yop1p and Sey1p function together in the process of membrane fusion. There are three major steps involved in membrane fusion. The first is tethering, which is dependent on YPT GTPases, and is mediated by peripheral membrane proteins. The second step is docking, or SNARE engagement, in which the integral membrane coiled coil SNARE proteins, located on both vesicle and target membranes, form very stable complexes, holding the vesicle in close proximity to the target membrane. Finally, there is fusion of the lipid bilayers (Pelham, 2001). Sequence homology searches indicate that many of the molecular components of the secretory pathway are conserved between plants and fungi. It may be that Yop1p and Sey1p act in the same or sequential steps in this pathway, which would explain the synthetic interaction between the two.

**rhd3 Phenotype**

In plants, root hairs serve to increase the surface area of roots for increased water and mineral absorption from the soil. They are extensions of single cells, and are an extreme example of polarized cell expansion. This growth takes place at the very tip, and is made possible by the delivery of cell wall components to the expanding zone via membrane-bound vesicles (Gilroy and Jones, 2000). When RHD3, the Arabidopsis homolog of SEY1, is mutated, the root hairs become short and wavy (Galway et al., 1997). Closer examination reveals an accumulation of vesicles in the root hair tip, indicating a problem with vesicle fusion (Galway et al., 1997). It is interesting to note that although the Arabidopsis rhd3 mutant has a very noticeable phenotype, deletion of SEY1 in yeast results in no noticeable morphological abnormalities (Fig. 6C) or growth phenotype (Fig. 5).

**SEY1 Alleles**

Calcium has been established as an important player in both regulated and constitutive membrane fusion, although its exact role remains unclear. High levels of Ca\(^{2+}\) can rescue the temperature-sensitive ypt1-1 mutant at the nonpermissive temperature (Schmitt et al., 1988). It may be that high levels of calcium facilitate some step of membrane fusion, which could overcome the inefficiency of fusion caused by the yop1Δ/sey1-3 synthetic interaction. The yop1Δ/sey1-3 mutant is sectoring when grown on media containing additional calcium, suggesting that the sey1-3 allele maintains some level of activity, and that Sey1p acts in some process involving calcium.

The SEY1-4 allele is conditional because it has sufficient activity at 37°C to allow sectoring, but not at room temperature. It is somewhat surprising that this allele has any activity because the mutation causes a truncation resulting in the loss of more than 60% of the predicted protein. Because the other three recovered alleles have lesions in the first quarter of the coding sequence, it is suggested that the N-terminal region is the most important section of the protein. This is where the putative GTP-binding motifs are located. In fact, the sey1-1 lesion is very close to the first GTP-binding motif, and the sey1-2 lesion disrupts the second GTP-binding motif.

**Yop1p N Terminus Is Not Required for Function**

It has been previously shown that Yop1p and Yip1p interact physically. Two-hybrid data show that the N-terminal 17 amino acids of Yop1p are necessary and sufficient for this interaction (Calero et al., 2001). Because YIP1 is essential for viability, whereas YOP1 is not, it is clear that the direct physical interaction between these two proteins is not necessary for the function of Yip1p. Because no biochemical function has been determined for Yop1p, it is not possible to directly assay for activity of the protein. However, the yop1Δ/sey1 sect− mutants provide a useful background for performing functional assays for Yop1p activity. The ability of the YOP1 N-terminal deletion mutant to restore sectoring to the yop1Δ/sey1 double mutants clearly demonstrates the activity of the truncated protein. Based on this observation, it can be inferred that the direct physical interaction between Yop1p and Yip1p is not required for the function of Yop1p. Large-scale two-hybrid analyses have identified proteins that interact with both Yip1p and Yop1p, including Yif1p and YLR324W (Ito et al., 2000, 2001a, 2001b; summarized in Fig. 9). It is possible that these four proteins form a complex, and the elimination of the direct interaction between Yip1p and Yop1p is not sufficient to disrupt the complex. Although no two-hybrid interactions involving Sey1p have been reported, an interesting possibility is that Sey1p somehow acts to stabilize this complex and that in a yop1Δ background, mutation of SEY1 may be sufficient to disrupt its formation. Alternatively, it is possible that the association of Yip1p and Yop1p is simply not functionally important.

**Yop1p Interactions with Yip1p and GTPases**

Calero et al. (2001) have recently demonstrated a physical interaction between Yop1p and Yip1p (Ypt1p-interacting protein) and the GTPases Sec4p, Ypt6p, and Ypt7p. In addition, Yop1p had previously been in a two-hybrid screen with Ypt1p (http://genome-www.stanford.edu/Saccharomyces/; Yang et al., 1998). The YPT family of proteins includes
rab-like GTPases that play important roles in vesicular trafficking. Their function has been reviewed recently (Segev, 2001a, 2001b). Ypt GTPases cycle between GTP- and GDP-bound forms. The GTP to GDP switch is accomplished through GTP hydrolysis, whereas the GDP to GTP switch is accomplished by nucleotide exchange. Four classes of proteins regulate this cycling: GAP (GTPase-activating protein), GEF (guanine nucleotide exchange factor), GDF (guanine dissociation factor), and GDI (GDP dissociation inhibitor). Vesicular fusion is mediated by the GTP-bound form of Ypt, so this form is considered active, whereas the GDP-bound form is considered inactive. As such, interacting factors involved in the formation or maintenance of GTP-bound Ypt (GEF and GDF) are positive regulators of Ypt function, whereas those that favor the GDP-bound form (GAP and GDI) are negative regulators of Ypt. Because GAP and GDI are important in the recycling of Ypt back to the membrane of the donor compartment, they have a positive role as well (Segev, 2001a, 2001b).

Although it is clear that Yop1p is involved in the process of membrane fusion, it is not clear why this protein is up-regulated by environmental stress. It has been reported that yeast and plants change the lipid composition of their plasma membrane in response to environmental conditions (Alexandre et al., 1994; Sharma et al., 1996; Smaoui and Cherif, 2000; Hamrouni et al., 2001). The newly synthesized lipids are in large part delivered to the plasma membrane by transport vesicles (Holthuis et al., 2001). It is possible that the cell will regulate components of the vesicular transport machinery in response to environmental stress to hasten the changes in membrane lipid composition.

Alternatively, Yop1p may be a negative regulator of Ypt proteins, which is suggested by the observation that overexpression of YOP1 blocks secretion. This seems a likely function of HVA22, the barley homolog of Yop1p, considering its regulation and the physiology of the barley seed. The primary function of the barley aleurone layer is the secretion of hydrolytic enzymes into the endosperm. Under favorable conditions, the phytohormone GA is produced by the embryo of the seed, and stimulates the production of hydrolytic enzymes by the aleurone layer. These are secreted into the endosperm, releasing sugars and amino acids to support the growth of the germinating embryo. If conditions become unfavorable, the phytohormone ABA is produced and antagonizes the action of GA (Lovegrove and Hooley, 2000). HVA22 was initially identified as an ABA-induced transcript in barley aleurone layers. If the antagonistic actions of ABA and GA serve to regulate secretion, a reasonable possibility is that HVA22 acts as a negative regulator of secretion, and may block secretion at elevated levels of expression. In vegetative tissues, GA promotes growth and elongation of tissues, whereas ABA blocks that action. In this case, ABA could be acting to block the expansion of cell membranes and the delivery of cell wall components as a mechanism to stop growth.

Transgenic Arabidopsis containing T-DNA disruptions of the five known HVA22 homologs (ATHVAA2a–ε) have been obtained recently. In addition, RNAi lines designed to knock down expression ofATHVAA2a and ATHVAA2d, as well as plants expressingATHVAA2a and ATHVAA2d under the control of the 35S promoter, have been generated. These plants are all viable, and more detailed phenotypic characterization is under way (N. Chen, personal communication).

### MATERIALS AND METHODS

#### Sequence Analyses

Amino acid sequences were aligned by MegAlign (DNA Star Inc., Madison, WI). Identification of similar residues was determined by SeqVu version 1.0 (The Garvan Institute of Medical Research, Sydney) using 90% on the Goldman, Eisenberg, Stelitz scale. Kyte-Doolittle hydrophilicity plots (Kyte and Doolittle, 1982) were generated by Protein (DNA Star Inc.) using a 9-amino acid window.

#### Strains, Growth Media, and Growth Conditions

Yeast (Saccharomyces cerevisiae) strains were grown in 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) Glc (YPD), or synthetic media supplemented with the appropriate nutrients. Growth curve experiments were performed by inoculating 30 mL of media with 100 µL of stationary phase culture that had been grown at room temperature. Three independent cultures for each strain were inoculated, and cell density determined by reading the A_600 determined by making a dilution of the culture sufficient to keep the spectrophotometer reading below 0.5. Yeast transformation was done by the lithium acetate method (Ito, 1983).

#### Deletion of the YOP1 Coding Sequence

The KANmx cassette was amplified with the primers 5'-TTGG-TAGTGGAAAAACAATATAACGACATAACGCCATCTAACAACCAT-GAACCCTGGTACCC3' and 5'-GGACACAAAAGGAGAGTTTGATTTGAGGGCTGATCGAGATTAGGTTGCGGCCGTACGATCAG-3'. The product was transformed into w303a/a and transformants selected for on YPD plates containing 100 µg mL−1 genetin (Sigma, St. Louis). Several transformants were screened by PCR to confirm the presence of the cassette at the YOP1 locus. One of the positive clones was designated ABY15. This strain was sporulated and dissected to create the strain ABY16.

#### DNA Constructs and Manipulations

Sequencing was done with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

The YOP1 genomic clone was constructed by gap repair. Plasmid p58W986 (ADE3 URA3 CEN; Murphy et al., 1996) was cut with NolI, treated with Klenow, and religated to destroy the NolI site, creating pABS28. The YOP1 genomic region was amplified with the primers 5'-ATATAATGGATTCCCTGGAAAACACTGTTGAGGCC3' and 5'-TATATAGGATCCTCTTCCAC-3'. The PCR product was cloned into the TA cloning vector pCRII (Invitrogen, Carlsbad, CA), making pAB21. The resulting plasmid was cut with BstBI and NolI to drop out the YOP1 coding sequence but leave behind the flanking genomic sequence. A linker fragment made by annealing primers 5'-CGAATTCGCCGGCCACA3' and 5'-TATATGGATCCTCTTCCAC-3' was cloned into pABS28, which had been cut with BstBI and NolI to introduce a NolI site, making pAB22. The BamHI insert from this plasmid was ligated into pABS28 to make pABS29. This plasmid was cut with NolI.
and transformed into yeast strain w303-1a to create a high-fidelity YOP1 clone by gap repair. The resulting plasmid (pABS30) was recovered by plasmid rescue, transformed into Escherichia coli, and checked by restriction digestion. The Yop1p N-terminal deletion construct was made by introducing restriction sites to the genomic clone by PCR. The promoter region was amplified using the primers 5'-ATATATGATCTCTTGAAAACCTGTTGAGCC-3' and 5'-CATGCCATGGTTGGATGT GCGTATG-3'. The second exon and 5'-untranslated region were amplified with the primers 5'- CATGCATGGTAGCATCCTGATATTAG-3' and 5'-ATTATAGTACCTCTGATTAATG-3'. The resulting products were cut with NcoI and BamHI for cloning into the vector pRS315 (LEU2 CEN).

** Yeast Mutagenesis and Screening for Synthetic Enhancement Mutants **

ABY16 was mated to YCH128, sporulated, and dissected to make the strains ABY29 and ABY30. These were both transformed with plasmid pABS30 (YOP1 URA3 ADE3 CEN) to create the strains ABY41 and ABY42. When grown on nonselective plates, these strains produce segregating colonies. Overnight cultures of yeast strains ABY41 and ABY42 grown in synthetic complete minus uracil media were used to inoculate 100 mL of synthetic complete minus uracil media. The cultures were grown to early log phase. Each culture, 5 x 10^5 cells were harvested and washed twice with 10 mL of 50 mM potassium phosphate buffer, pH 7.0. The cells were resuspended in 10 mL of potassium phosphate buffer. Thirty milligrams of ethyl methanesulfonate was added to 1 mL of each sample, then incubated for 70, 80, 90, and 100 min. At the end of the incubation time, 1 mL of 10% (v/v) filter-sterilized fresh sodium thiosulfate was added to each tube. The samples were washed twice with 10 mL of water and resuspended in 1 mL of water. Dilutions were plated on rich media to determine survival rate. Cells from the 80-min treatment had an approximately 20% survival rate, and were used for screening. Approximately 120,000 total colonies were screened for sech- phenotype, sech- strains were isolated and subjected to complementation group analysis and tested for YOP1 and URA3 depen- dence. For complementation group analysis, all ABY41-derived strains were cross to all ABY42-derived strains, and scored for sechering. Four mutants belonging to one complementation group were found to become sech- upon transformation with pABS33 (YOP1 LEU2), but not upon transformation with pABS32 (URA3 LEU2), indicating dependence upon YOP1.

** Cloning of YOR165W **

sech- mutant ABY51 was transformed with a genomic library (LEU2 CEN), and transformants were screened for sechering. Selecting colonies were picked and restreaked on C-LEU plates. Individual colonies were used to grow overnight cultures for plasmid recovery. The inserts of recovered plasmids were end sequenced to determine the genomic region they con- tained. Subcloning and deletion analysis was carried out to identify ORF YOR165W as the complementing gene.

** Cloning of YOR165W Alleles **

Plasmid pABS34 (SEY1 LEU2) was cut with SnaBI, releasing an 8,928-bp fragment containing the SEY1 locus. The plasmid was religated to make pABS45. This plasmid was cut with SnaBI and transformed into the mutants to clone the SEY1 locus by gap repair. Recovered plasmids were checked by restriction digest for the presence of SEY1, and sequenced. sey1-2 was resistant to cloning by this method, and therefore was cloned by PCR using the primers 5'-GAGTTTCGCTTGTACAGCATTAGAT-3' and 5'-TGAACA-ATTTGGGAGACTGTATT-3'. Clones from two independent PCR reac- tions were sequenced, and found to contain the same lesion.

** EM **

Yeast cultures were grown in YPD media to log phase. White colonies derived from the conditional mutant ABY53 were isolated at 37°C. Liquid media was inoculated with a well-isolated white colony and the culture grown at 37°C to log phase, then shifted to 25°C for 2 h. Samples were washed with water, and then resuspended in 2.5% (v/v) glacialdehyde in 40 mM phosphate buffer, pH 6.5, and 0.5 mM MgCl2. Samples were incubated at room temperature for 2 h, then washed twice with 5 mL of 0.1 M PO4 citrate buffer, pH 5.8. To remove cell walls, cells were treated with zymolysate for 2 h. Samples were then washed twice with 5 mL of 1 M NaOAc pH 6.2. Post-fixation was done with 2% (w/v) osmium tetroxide followed by 1% (w/v) uranyl acetate and the samples were embedded in Spurr's resin.

** Invertase Assays **

Yeast cultures were grown in YP media supplemented with 5% (w/v) Glc to log phase. 1 x 10^8 cells were harvested, washed twice with water, and resuspended in YP media containing 0.05% (w/v) Glc. After 3 h of shaking, NaNO3 was added to 10 mM, the samples were aliquoted into five 1-mL samples, washed with 10 mM NaNO3, then resuspended in 10 mM NaNO3. Each sample was divided in two for total and external invertase assays. External invertase activity was measured from intact cells. For total activity, Triton X-100 was added to 0.2% (v/v), and the samples were frozen and thawed twice on dry ice to lyse the cells. For the assays, a 50-μL aliquot of cells was mixed with 100 μL of 0.2 mM sodium acetate, pH 5.1, at 37°C. To start the reaction, 50 μL of 0.5 mM Suc (Plasnsted Laboratories, Waukegan, IL) was added to the samples. After 20 min, the reaction was stopped by adding 300 μL of 0.2 mM K2HPO4. After mixing, 100 μL of this mix was added to 400 μL of 0.2 mM K2HPO4 in a glass tube and immediately boiled for 3 min. A 2-mL aliquot of a solution containing 0.1 mM potassium phosphate (pH 7.0), 20 μg mL^-1 Glic oxidase, 2.5 μg mL^-1 peroxidase, and 150 μg mL^-1 O-dianisidine was added to each sample and incubated at 37°C. After 30 min, the reaction was stopped by adding 2 mL of 6 M HCl and absorption was read at 540 nm.

** Two-Hybrid Assays **

The YOP1 cDNA was amplified from total RNA by reverse transcriptase-PCR using primers to add restriction sites for cloning. For cloning into the pBD-GAL4 vector (Stratagene), the primers 5'-CGGCGCATCATGTCGCAATAGC-3' and 5'-TAACTGCAGTTAATGAACAGAAG-3' were used to add SaI and PstI sites. For cloning into the pGAD424 vector (CLONTECH Laboratories, Palo Alto, CA), the primers 5'-TGGGATCCATGTCGCAATAGC-3' and 5'-TAACTGCAGTTAATGAACAGAAG-3' were used to add BamHI and PstI sites. Constructs were transformed into YRG-2. Transformants were streaked out on C-Leu-Trp, and -His and scored for growth after 5 days at 30°C.

** ACKNOWLEDGMENTS **

The authors thank Susan Wente, Kathy Iovine, Mirella Bucci, Albert Ho, and Kathy Ryan for protocols, reagents, and technical advice; Mike Veith for technical assistance with EM; Ken Blumer for use of his tetrads dissection apparatus; and members of the Ho lab for technical assistance, discussion, and comments on the manuscript.

Received April 26, 2002; returned for revision June 3, 2002; accepted July 19, 2002.

** LITERATURE CITED **


Involvement of the Stress-Induced Gene HVA22 in Secretion


Nigavekar SS, Cannon JF (2002) Characterization of genes that are synthetically lethal with ade3 or leu2 in Saccharomyces cerevisiae. Yeast 19: 115–122


Segev N (2001b) Ypt/Rab GTPases: Regulators of Protein Trafficking. Science’s STKE, http://stke.sciencemag.org/cgi/content/full/OC_sigttrans; 2001/100/re11


