Functional Characterization of the *Saccharomyces cerevisiae* *VHS3* Gene

A REGULATORY SUBUNIT OF THE Ppz1 PROTEIN PHOSPHATASE WITH NOVEL, PHOSPHATASE-UNRELATED FUNCTIONS*

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The yeast gene *VHS3* (YOR054c) has been recently identified as a multicopy suppressor of the G_1/S cell cycle blockade of a conditional *sit4* and *hal3* mutant. Vhs3 is structurally related to Hal3, a negative regulatory subunit of the Ser/Thr protein phosphatase Ppz1 important for cell integrity, salt tolerance, and cell cycle control. Phenotypic analyses using *vhs3* mutants and overexpressing strains clearly show that Vhs3 has functions reminiscent to those of Hal3 and contrary to those of Ppz1. Mutation of Vhs3 His[459], equivalent to the supposedly functionally relevant His[90] in the plant homolog AtHal3a, did not affect Vhs3 functions mentioned above. Similarly to Hal3, Vhs3 binds *in vivo* to the C-terminal catalytic moiety of Ppz1 and inhibits *in vitro* its phosphatase activity. Therefore, our results indicate that Vhs3 plays a role as an inhibitory subunit of Ppz1. We have found that the *vhs3* and *hal3* mutations are synthetically lethal. Remarkably, lethality is not suppressed by deletion of *PPZ1*, *PPZ2*, or both phosphatase genes, indicating that it is not because of an excess of Ppz phosphatase activity. Furthermore, a Vhs3 version carrying the H459A mutation did not rescue the synthetically lethal phenotype. A conditional *vhs3 tetO:HAL3* double mutant displays, in the presence of doxycycline, a flocculation phenotype that is dependent on the presence of Flo8 and Flo11. These results indicate that, besides its role as Ppz1 inhibitory subunit, Vhs3 (and probably Hal3) might have important Ppz-independent functions.

The *Saccharomyces cerevisiae* Ppz Ser/Thr protein phosphatases, encoded by genes *PPZ1* and *PPZ2* (1–3), are characterized by a C-terminal half closely related to type 1 phosphata-

ses (see Ref. 4 for a recent review). These phosphatases are involved in several cell processes. They interact functionally with the protein kinase C-activated MAP[1] kinase pathway and thus play a role in cell wall integrity (2, 5), regulate salt tolerance (6), and control cell cycle at the G_1/S transition (7, 8). In all these cases, Ppz1 has a more prominent role, as denoted by the observation that cells lacking Ppz2 display a wild-type phenotype, unless *PPZ1* has been also deleted. Recent evidence indicates that most of the phenotypes associated with the absence or the overexpression of the Ppz phosphatases are a consequence of the inhibitory effect that these phosphatases exert on the function of the Trk1/Trk2 potassium transporters (9). In addition, it has been postulated a negative role for Ppz1 on the calcineurin pathway, which would explain the increased expression of the *ENA1* Na$^+$-ATPase in *ppz1* cells and, at least in part, the salt tolerant phenotype of the mutant strain (10).

Ppz1 is negatively regulated by Hal3/Sis2, which binds to the C-terminal catalytic moiety of the phosphatase and inhibits its activity (11), although the mechanism of inhibition is still unknown. *HAL3/SIS2* was identified independently by two laboratories: as a gene able to confer saline tolerance when overexpressed (12), and as a multicopy suppressor of the growth defect of a *sit4* mutant (13). Sit4 is a Ser/Thr protein phosphatase (14) required for proper passage from G_1 to S phase (15, 16). The current evidence suggests that Hal3 regulates most (if not all) the functions of Ppz1. Therefore, overexpression of *HAL3* in an *slt2/mpk1* MAP kinase mutant aggravates the lytic defect of this strain (11), thus mimicking the effect of deletion of *PPZ1*. Similarly, overexpression of *HAL3* confers salt tolerance (and increases *ENA1* expression), and mutation of the gene results in salt sensitivity, in a Ppz-dependent fashion (10, 11). Finally, it is known that the *sit4* and *hal3* mutations are synthetically lethal because of a G_1/S blockade (13, 17), and this phenotype is suppressed by disruption of *PPZ1* (8).

In a recent work (18) we reported the use of a conditional *sit4Δ tetO:HAL3* strain to screen for multicopy suppressors of the G_1 blockade suffered by this strain under non permissive conditions (presence of doxycycline). Among the several ORFs identified, YOR054c (renamed as *VHS3*) has our immediate attention because it encoded an acidic, 674 residue protein displaying a substantial sequence similarity (49% identity) with Hal3. In this work we characterize the biological role of Vhs3 and demonstrate that in addition to acting as a negative

[1] The abbreviations used are: MAP, mitogen-activated protein; GST, glutathione *S*-transferase; 5-FOA, 5-fluoro-orotic acid; ORF, open reading frame.

TABLE I

Yeast strains used in this work

Name	Relevant genotype	Source/Ref.
JA100	MATa *ura3–52 leu2–3,112 his4 trp1–1 can-1r*	11
JA110	JA100 *sit4::TRP1*	8
JC002	JA100 *sit4::TRP1* prom*tet0::HAL3*	17
JC133	JA100 *sit4::TRP1 vhs3::URA3*	This work
JC010	JA100 *mpk1::LEU2*	60
MAR20	JA100 *mpk1::LEU2 hal3::LEU2*	This work
MAR9	JA100 *mpk1::LEU2 vhs3::URA3*	This work
JA104	JA100 *hal3::LEU2*	11
MAR88	JA100 *hal3::kanMX*	This work
MAR79	JA100 *vhs3::URA3*	This work
MAR107	JA100 *hal3::kanMX vhs3::nat1* [YEplac195-*HAL3*]	This work
MAR109	JA100 *hal3::kanMX vhs3::nat1 ppz1::LEU2* [YEplac195-*HAL3*]	This work
MAR110	JA100 *hal3::kanMX vhs3::nat1 ppz1::LEU2 ppz2::TRP1* [YEplac195-*HAL3*]	This work
EDN75	JA100 *ppz1::KANMx*	24
JC001	JA100 prom*tet0::HAL3*	This work
MAR24	JA100 prom*tet0::HAL3 vhs3::URA3*	This work
MAR80	JA100 prom*tet0::HAL3 flo11::TRP1*	This work
MAR81	JA100 prom*tet0::HAL3 vhs3::URA3 flo11::TRP1*	This work
MAR82	JA100 prom*tet0::HAL3 flo8::TRP1*	This work
MAR83	JA100 prom*tet0::HAL3 vhs3::URA3 flo8::TRP1*	This work
MAR84	JA100 prom*tet0::HAL3 ste12::TRP1*	This work
MAR85	JA100 prom*tet0::HAL3 vhs3::URA3 ste12::TRP1*	This work
MAR86	JA100 prom*tet0::HAL3 tec1::TRP1*	This work
MAR87	JA100 prom*tet0::HAL3 vhs3::URA3 tec1::TRP1*	This work
AGS1	JA100 prom*tet0::HAL3 mss11::TRP1*	This work
AGS2	JA100 prom*tet0::HAL3 vhs3::URA3 mss11::TRP1*	This work
1788	MAT**a**/a *ura3–52 leu2–3,112 his4 trp1–1 can-1r*	D. Levin
MAR6	1788 *hal3::LEU2/HAL3 vhs3::URA3/VHS3*	This work
AGS4	1788 *hal3::LEU2/HAL3 vhs3::kanMX/VHS3*	This work
MAR26	1788 *hal3::LEU2/HAL3 vhs3::URA3/VHS3 ppz1::KAN/PPZ1*	This work
MAR23	1788 *hal3::LEU2/HAL3 vhs3::URA3/VHS3 ppz2::TRP1/PPZ2*	This work
MAR27	1788 *hal3::LEU2/HAL3 vhs3::URA3/VHS3 ppz1::KAN/PPZ1 ppz2::TRP1/PPZ2*	This work
MAR112	1788 *hal3::LEU2/HAL3 vhs3::nat1/VHS3 ppz1::KAN/PPZ1 ppz2::TRP1/PPZ2*	This work
MAR113	*MAT**a** hal3::LEU2 vhs3::nat1 ppz1::KAN/ ppz2::TRP1* [YEplac195-*HAL3*]	This work
MAR115	*MAT**a** hal3::LEU2/HAL3 vhs3::kanMX/VHS3* [YEplac195-*VHS3*]	This work
MCY3000	FY250 *glc7–T152K*	32
DBY746	*MATα ura3–52 leu2–3,112 his3-Δ1 trp1-Δ239*	D. Botstein
EDN4	DBY746 *hal3::LEU2*	61
EDN2	DBY746 *ppz1::TRP1*	10
EDN85	DBY746 *ppz1::TRP1 ppz2::KAN*	10
MAR11	DBY746 *vhs3::URA3*	This work

regulatory subunit of the Ppz1 protein phosphatase, this protein may also have other important functions.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—Yeast cells were grown at 28 °C in YPD medium (10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter dextrose) or, when indicated, in synthetic minimal or complete minimal medium (19). The relevant genotype of the strains described in this work can be found in Table I.

Gene Disruption and Plasmid Construction—Disruption of the *VHS3* gene was done by cloning a 3.6-kbp KpnI/EcoRI genomic fragment, encompassing the entire gene, into the same sites of plasmid pUC19, to yield pUC19-VHS3 and replacing a 1.05-kbp NheI/SnaBI fragment with a NheI/SmaI 1.1-kbp fragment from plasmid YDp-U (20), containing the *URA3* marker. The disruption cassette is recovered by digestion with KpnI/EcoRI and used to transform yeast cells. Disruption of *VHS3* with the *nat1* marker was accomplished as follows. The 1.29-kbp PvuII/SpeI fragment from plasmid pAG25 (21), containing the *nat1* gene from *Streptomyces noursei* was used to replace the 1.05-kbp SnaBI/NheI fragment of plasmid pUC19-VHS3. The deletion cassette was released by digestion with AfeI/PvuII and used to transform the appropriate strains. Positive clones were selected in the presence of nourseothricin (21). Disruption of *VHS3* with the *kan*MX marker (from nucleotides −40 to 2050, relative to the initiating Met codon) was accomplished by the short flanking gene replacement technique (22). The *HAL3* gene was disrupted with the *LEU2* marker as previously described (12). To construct strain MAR88 (*hal3::kanMX*), genomic DNA from the appropriate deletion mutant in the BY4741 background (23) was used to amplify the *HAL3* genomic locus using oligonucleotides spanning from positions −108 to +2150, and the amplification fragment used to transform the wild-type strain JA100. Deletion of the *PPZ1* gene with the *kanMX* marker has been reported previously (24). Deletion of *PPZ1* with the *LEU2* marker was done by replacing a 1.6-kbp XhoI/StuI

fragment from plasmid YEp181-Ppz1 (7) with the SmaI/SalI 1.7 kbp *LEU2* marker from plasmid YDp-L (20). The PstI/NdeI 2.5 kbp cassette is used to transform cells. Deletion of *PPZ2* with a *TRP1* marker was performed as described in Ref. 7. The use of the cassette *tetO:HAL3* was described previously in Ref. 17.

Deletion of the *FLO11/MUC1* gene was performed as follows. A 5.2-kbp region of the *FLO11* genomic locus spanning the entire ORF, plus 484 nucleotides upstream and 761 nucleotides downstream, was amplified by PCR and cloned into the XbaI/EcoRV sites of pBluescript to give plasmid pBS-*FLO11*. Then, a 3.6-kbp HincII/PstI fragment of the ORF was replaced by the 0.85-kbp *TRP1* marker, recovered from plasmid YDp-W (20) by digestion with SmaI/PstI, to yield plasmid pBS-*FLO11::TRP1*. This plasmid was digested with ApaI/SacI, and the resulting 2.5-kbp fragment was used to transform cells.

To construct the *flo8::TRP1* cassette, a 3.99-kbp fragment spanning from −998 to +2987 (referred to the ATG of the *FLO8* ORF) was amplified by PCR, digested with XbaI (an artificially added site) and EcoRV, and then cloned into the XbaI and HincII sites of pBluescript to give plasmid pBS-*FLO8*. An EcoRI/SalI 1.8-kbp fragment of the *FLO8* gene was replaced by the marker *TRP1* (0.85 kbp), recovered from plasmid YDp-W. The resulting construct, pBS-*flo8::TRP1*, was digested with NdeI, and cells were transformed with the 2.65-kbp fragment released.

To disrupt the *STE12* gene, a 3.7-kbp fragment (from −970 to +2700 relative to the initiating codon) was amplified by PCR including added PstI and EcoRI sites. After digestion with the indicated enzymes, it was inserted into pUC19 generating pUC19-*STE12*. Then, a HincII/BamHI 1.6-kbp fragment was replaced by the marker *TRP1* (obtained by digestion of YDp-W with SmaI/BamHI). This plasmid, called pUC19-*ste12::TRP1* was digested with SacI, and the 1.75-kbp *ste12::TRP1* cassette used to transform the appropriate strains.

Disruption of the *TEC1* gene was made as follows. First, a 3.0-kbp fragment from positions −1050 to +1950 (referred to the ATG codon)

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Vhs3      1  ----MINKSSLKNNRKGVASNTLSGAEQANIGSSAMPDTNSTGPFSSVSSLDTPVVRKSI
ScHal3    1  MTAVASTSGKQDADHNQSIECPRFSRGQKEILLDHEDAKGKDSIINSPVSGRQSISPTLS
AtHal3a   1  ------------------------------------------------------------

Vhs3     57  SPTGSQTKSIMNASGTSGAVVSNTPEPGLKRIETVTFSDPKLGSLRSDVEQTPPNQVARQ
ScHal3   61  NAITLTTKSIMNATGTSGAVVSNTPEPGLKRVPAVTFSDLKQ------------------
AtHal3a   1  ------------------------------------------------------------

Vhs3    117  SSEKKATSVHIAAEGANQGRNLKDINTKVPKDGEASASSFSTPTSILSNADMGNNISSLL
ScHal3  103  --------------------QQKQDSLTQLKNDSPRTKSPNSNPAPVSNSIPGNHAVIPNH
AtHal3a   1  ------------------------------------------------------------

Vhs3    177  AKKLSFTGGIDSILNSDNSSDSPRKEHPHFYVEDPLHTPSVRSRSNSTSPRPSVVVNTFN
ScHal3  144  TNTSRTTQLSGSPLVNEMKDYDPKKKDSALKIVDTMKPDKIMATSTPISRENNKVTAKAP
AtHal3a   1  ------------------------------------------------------------

ScHal3  204  TSITILRKEDAQLQANNVSGQINVRSIPEETPVKQSVIPSIIPKRENSKNLDPRLPQDDGK
Vhs3    237  PINIER-----EGSISKIGEPTLLESVLEEAVSPNAVSNPIKRENIMTNMDPRLPQDDGK
AtHal3a   1  -------------------------MENGKRDRQDMEVNTTPRKFR----

Vhs3    292  LHVLFGATGSLSVFKLKHMIRKLEEIYGRDKICIQVILTNSATKFEAMKYMRKNKKQHNS
ScHal3  264  LHVLFGATGSLSVFKIKPMIKKLEEIYGRDRISIQVILTQSATQFFQRYTKKIIKSSEK
AtHal3a  22  --VILAASGSVAAIKFGNICHCFTEWA-----EVRAVVTKSSLHFLLK-----------

Vhs3    352  IDTSFNSTNSNAGNITGNKKKVASLEKFSIQKTSSNSAASQTNNKQEEEKQMASTTGFPS
ScHal3  324  LNKMSCYESIPATPVTP-------------------------------------------
AtHal3a  63  ------------------------------------------------------------

Vhs3    412  TLGGSRTYSNSSNVVSQHPQLELPAHIQFWTDQDEWDVWRQRTDPVLHIELRRWADILVV
ScHal3  341  ----------LPGQCNMAQVVELPPHIQLWTDQDEWDAWKQRTDPVLHIELRRWADILVV
AtHal3a  63  --------------------ISLPQEVTLYTDEDEWSSWNKIGDPVLHIELRRWADVLVT

Vhs3    472  APLTANTLAKIALGLCDNLLTSVIRAWNPTFPIFLAPSMGSGTFNSIMTKKHFRIIQEEM
ScHal3  391  APLTANTLSKIALGLCDNLLTSVIRAWNPSYPILLAPSMVSSTFNSMMTKKQIQTIKEEM
AtHal3a 103  APLSANTLGKIAGGLCDNLLTCIIRAWDYTKPIFVAPAMPNTLMWNNPFTERHLLSLDELG

Vhs3    532  PWVTVFKPSEKVMGINGDIGLSGMMDANEIVGKIVVKLGGYPDVSAGKEEEEDEDNDEED
ScHal3  451  SWVTVFKPSEKVMDINGDIGLGGMMDWNEIVNKIVMKLGGYPKN---NEEEDDDEDEEED
AtHal3a 163  ---ITLIPPIKKRLACGDYGNGAMAEPSLIYSTVRLFW--------------------P

Vhs3    592  DNKKNDTGGKDEDNDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDDD
ScHal3  508  DDEEEDTEDKNENNNDDDDDDDDDDDDDDDDDDDDDDDDDEDEDEAETPGIIDKHQ----
AtHal3a 199  SQAHCQTGGTS------------------------------------------------
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FIG. 1. **Pairwise comparison using the ClustalW program of the Vhs3, *S. cerevisiae* Hal3 (ScHal3), and *A. thaliana* Hal3a (AtHal3a) proteins.** *Black boxes* indicate identical amino acids, and *gray boxes* denote conserved changes. The *asterisks* indicate the conserved His residue (His[459] in Vhs3) and the non-conserved Cys residue (Cys[175], present only in AtHal3a). The *line* shows the HVXF motif.

was amplified by PCR using a 3′-primer with a KpnI artificial site. The fragment obtained by digestion with PstI and KpnI was cloned into pUC19, yielding plasmid pUC19-*TEC1*. Then, the 0.92-kbp XbaI/StuI fragment was replaced by the SmaI/NheI fragment of YDp-W, which contains the *TRP1* marker and the resulting construct (pUC19-*tec1*::*TRP1*) digested with SphI and XhoI to produce the *tec1*::*TRP1* cassette (2.95 kbp) employed for yeast transformation.

The *MSS11* gene was disrupted with the *TRP1* marker as follows. A fragment from −950 to +2742 was amplified by PCR with an added HindIII restriction site, digested with BamHI/HindIII and cloned into the same sites of plasmid pUC19 to give pUC19-*MSS11*. The construct was cleaved by XhoI/HincII and the 0.89-kbp DNA fragment obtained was replaced with a 0.85-kbp *TRP1* marker, previously released from plasmid YDp-W by digestion with SalI/SmaI. The final construct, pUC19-*mss11*::*TRP1*, was digested with SnaBI/KpnI and the 2.47 kbp fragment released was used for yeast transformation. All gene deletions generated in this work were confirmed by PCR.

For high copy expression of *HAL3*, the gene was recovered from plasmid YEp351-HAL3 (12) by digestion with EcoRI/HindIII and cloned into these sites of YEplac112 (*TRP1* marker) and YEplac181 (*LEU2* marker) vectors (25). High copy expression of *VHS3* was achieved by cloning a 3566-bp insert, starting from a KpnI site at position −776 from the ATG codon of *VHS3* and ending at the EcoRI site located 765 bp after the stop codon, at these sites of plasmids YEplac195 (*URA3* marker), YEplac181, or YEplac112 (*TRP1* marker). An identical cloning strategy using plasmid YCplac22 (*TRP1* marker) allowed low copy, centromeric expression.

The construction of a version of *VHS3* carrying a C-terminal 3×FLAG tag was as follows. An artificial SacI site right in front of the stop codon (which introduces the residues EL) was created by sequential PCR using external oligonucleotides that encompassed the ClaI and AfeI sites. The amplification fragment was digested with these enzymes and used to replace the 1.13-kbp ClaI/AfeI fragment of YEp195-VHS3. A 3×FLAG tag, with added SacI sites, was amplified by PCR from plasmid pCM220 (a gift of M. Aldea, Universitat de Lleida, Spain) and

then cloned into the previous construct in the appropriate orientation to yield YEp195-VHS3(3×FLAG).

Mutation of Vhs3 His[459] to Ala was made by sequential PCR. In a first step, the 1.13-kbp ClaI/AfeI fragment of *VHS3* gene was amplified in two separate reactions by using primers with the modification introduced to change His[459] to Ala (CA to GC). In the second step the entire ClaI/AfeI fragment was amplified, digested, and the product cloned into the ClaI/AfeI sites of YEp195-VHS3, to yield YEp195-VHS3(H459A). The mutated version of the gene was also cloned in the centromeric plasmid YCplac22 as a KpnI/EcoRI fragment.

To express in bacteria GST-Hal3 and GST-Vhs3 fusion proteins the *HAL3* and *VHS3* genes were amplified by PCR, with added EcoRI/XhoI sites, and cloned into plasmid pGEX6P-1 (Amersham Biosciences). The catalytic domain of Ppz1 (Δ1–344) amplified by PCR as described previously (7) was cloned into the SalI and HindIII sites of the pSP72 vector (Promega) to yield plasmid pSC2. The insert was recovered by digestion with BamHI and PvuII and cloned into the BamHI and SmaI sites of plasmid pGEX6P-1. The constructs for bacterial expression of GST-Ypi1 and GST-Glc7 have been previously described (26).

A *FLO11*-lacZ reporter plasmid (pFLO11-LacZ) was constructed by PCR amplification of the 3-kbp region 5′ of the ATG using primers with added BglII sites, following by cloning into the BamHI site of YEp367R (27). All fragments generated by PCR reactions (with the only exception of the *FLO11* promoter) were fully sequenced to confirm the absence of unwanted changes.

β-Galactosidase Assays—To evaluate the influence of high levels of *HAL3* or *VHS3* on *ENA1* expression, the different strains tested were transformed with plasmids YEpHAL3 or YEpVHS3, and then with plasmid pKC201, which contains the entire *ENA1* promoter fused to the β-galactosidase gene (28). β-Galactosidase assays were carried out as described in Ref. 10. Analysis of the *FLO11* promoter activity was carried out by introducing into the appropriate strains the *FLO11*-lacZ reporter described above. Cultures were grown overnight in selective medium, diluted up to an OD_{660} of 0.005 and then grown for 15 h at 28 °C in YPD medium, in the presence or the absence of 100 μg/ml
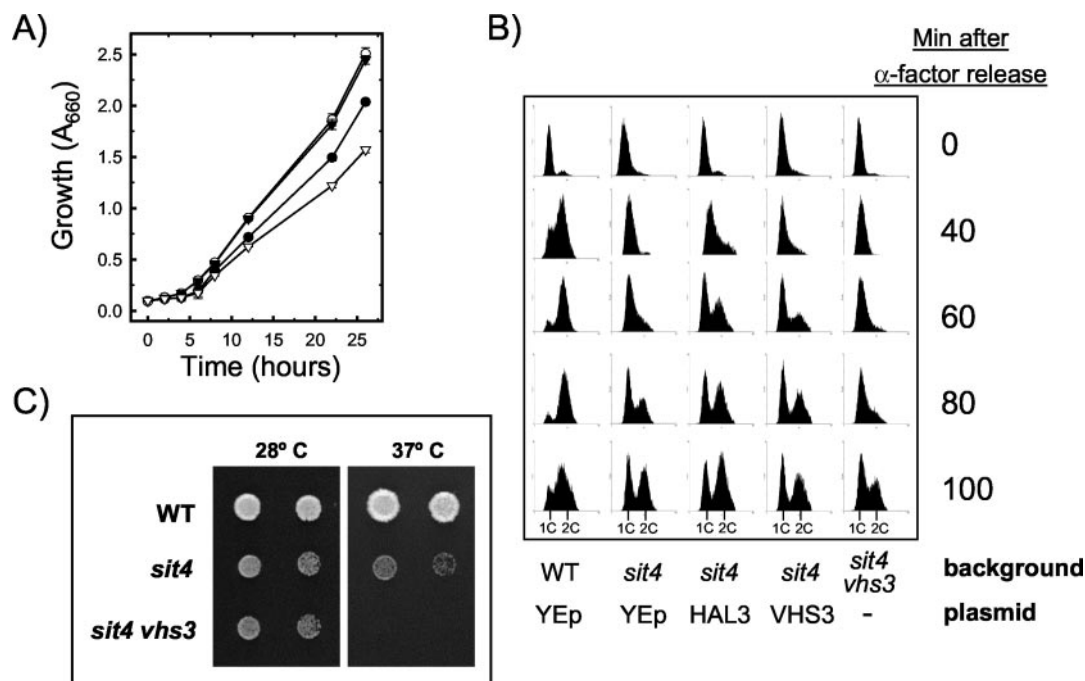
Fig. 2. **Effect of high copy expression of *VHS3* on *sit4* cells.** *A*, JA110 cells (*sit4*) bearing an empty plasmid (●), as well as carrying YEp195-HAL3 (○) or YEp195-VHS3 (▼) plasmids, and strain JC133 (*sit4 vhs3*, ▽) were inoculated on synthetic minimal medium lacking uracil at a OD$_{660}$ of 0.01, and growth was monitored for the indicated times. *B*, above-mentioned strains plus the wild-type (*WT*) strain JA100 bearing the indicated plasmids were arrested in G$_1$ by incubation with α-factor. The pheromone was washed out and entry into cell cycle monitored by the DNA content (Fig. 2) and budding index (not shown). *C*, indicated strains (two dilutions, 1:5) were grown at 28 or 37 °C on synthetic minimal medium. Growth was monitored after 3 days.

doxycycline (control cells received the same volume of the vehicle, a 50% ethanol solution). Then cells were collected and processed for β-galactosidase assay as above.

In Vitro and in Vivo Binding Assays—*In vitro* binding assays were performed as follows. GST-Ppz1(Δ1–344) was expressed in bacteria and bound to glutathione-agarose beads essentially as described previously (26). Cell extracts from strain EDN75 (*ppz1*Δ) were prepared as described (11) and 1 mg of total protein incubated with 50 μl of the affinity beads for 1 h at 4 °C. Washing and subsequent procedures were as in Ref. 11 except that after extensive washing the beads were resuspended in 100 μl of 2× SDS sample buffer and boiled. After a brief centrifugation, the sample (10 μl) was electrophoresed, transferred to membranes, and probed with anti-FLAG-antibodies (Sigma).

Expression in yeast of the GST fusion versions of the full-length and the catalytic domain of Ppz1 from the native *PPZ1* promoter was accomplished by transformation of strain EDN75 (*ppz1::kanMX*) with plasmids pYGST-C1Z1 and pYGST-C2Z1, respectively (11). Identification of *in vivo* binding of Vhs3 with the different Ppz1 versions was carried out essentially as previously described for Hal3 (11) except that cells were transformed in this case with plasmid YEp195-VHS3(3×FLAG) and bound proteins revealed with anti-FLAG antibodies.

In Vitro Phosphatase Assays—The effect of the diverse inhibitors on Ppz1 and Glc7 phosphatase activities were evaluated using bacterially expressed proteins. Conditions for expression and purification of the Ypi1, Ppz1(Δ1–344), and Glc7 fusion proteins have been previously reported (26). For expression of the phosphatases the growth medium did include 0.5 mM MnCl$_2$. GST-Hal3 and GST-Vhs3 were expressed in bacteria by induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 30 °C and purified essentially as described previously (26).

Ppz1 and Glc7 phosphatase activities were measured using *p*-nitrophenyl phosphate as in Ref. 26 with the following modifications: the concentration of substrate was 10 mM, 1 μg of Ppz1 and 2 μg of Glc7 were used, and the assays were carried out for 20 min. When inhibitors were included, they were incubated with the phosphatase at 30 °C for 5 min. The reactions were carried out in a final volume of 300 μl.

The protein phosphatase activity of Ppz1 was assayed as using the HA-tagged N-terminal domain of Reg1 as endogenous substrate. Crude yeast extracts were prepared as described (11), and the assay was performed essentially as described in Ref. 26, except that 2 μg of GST-Ppz1(Δ1–344) was used in the assay.

Other Techniques—Growth on plates (drop tests) or in liquid cultures was assessed as in Refs. 6 and 29, respectively. Yeast cells were arrested in G$_1$ with α-factor, and DNA content monitored by flow cytom-

etry as in Ref. 8. Budding index was determined by microscopic counting of at least 400 cells per point. Sporulation and tetrad dissection were done essentially as described in Ref. 19. Random spore analysis was performed as in Ref. 30. For 5-fluoro-orotic acid (5-FOA) selection, cells were grown overnight on YPD and around 1000 colony forming units plated in complete minimal medium (containing 50 μg/ml uracil) with 1 mg/ml 5-FOA.

Flocculation assays were performed as described by Bony *et al.* (31) with several modifications. Basically, yeast cells were inoculated in minimal medium at OD$_{660}$ 0.01 with or without 50 μg/ml of doxycycline and were incubated for 2 days. Then, cells were deflocculated and the OD$_{600}$ of the suspension measured. After the addition of calcium chloride (that induces flocculation), tubes were shaken at 28 °C (230 strokes/min) for 5 min and left standing for 4 min. A sample of the supernatant was then taken, made 0.21 M EDTA, and the OD$_{600}$ measured. The flocculation index is expressed as the ratio of the OD$_{600}$ between the fully deflocculated cell suspension and the EDTA-treated supernatant.

RESULTS

Functional Analysis of VHS3 Reveals Close Similarity with HAL3—*VHS3* was identified in our laboratory as a gene that, in high copy number, was able to rescue the G$_1$/S cell cycle blockade of a conditional *sit4 hal3* mutant. As the primary sequence of Vhs3 revealed a significant similarity with Hal3 (Fig. 1), we considered the possibility that Vhs3 could be functionally mimicking Hal3. To test this possibility, we transformed a *sit4* strain with a high copy plasmid bearing the *VHS3* gene. Overexpression of *VHS3* improved the slow growth phenotype of a *sit4*Δ strain similar to the *HAL3* gene (Fig. 2). This effect was also detected when cells were synchronized with α-factor, and recovery from the G$_1$ arrest was followed by monitoring DNA content (Fig. 2) and budding index (not shown). However, in cells arrested in G$_1$, the effect of overexpression of *VHS3* was weaker than that produced by *HAL3*. In contrast to the observation that deletion of *HAL3* is lethal in the absence of *sit4* (8, 13), we have been able to construct viable double *sit4 vhs3* strains. This strain, however, grows more slowly than the single *sit4* mutant under standard conditions and recovers very slowly from a α-factor-induced G$_1$ arrest. In
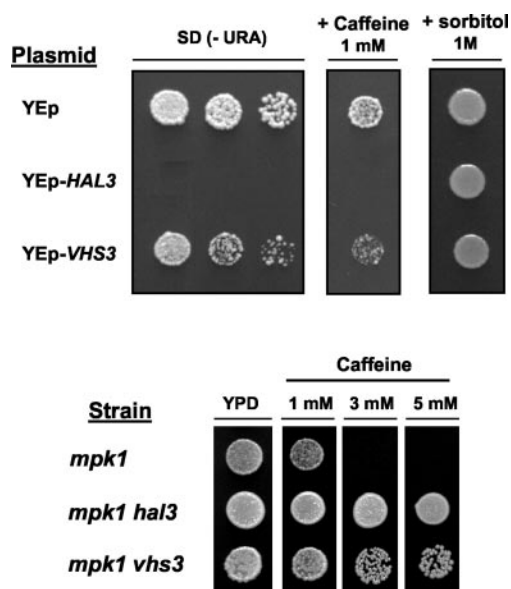
FIG. 3. **Effect of *VHS3* overexpression or deletion in a *slt2/mpk1* background.** *Upper panel*, strain JC010 (*mpk1Δ*) was transformed with the indicated plasmids and spotted on synthetic minimal (*SD*) plates lacking uracil (three dilutions of the culture) or on plates containing the indicated concentrations of caffeine or sorbitol. *Lower panel*, *HAL3* and *VHS3* genes were deleted in a JC010 background, and the sensitivity of the different strains to several concentrations of caffeine was tested. Growth was monitored after 60 h at 28 °C.

addition, mutation of *vhs3* greatly enhances the characteristic temperature-sensitive growth defect of the *sit4* mutation (Fig. 2).

An excess of Hal3 is deleterious for a cell lacking the Slt2/Mpk1 MAP kinase (11). We found that overexpression of *VHS3* in a *slt2/mpk1* strain grown in synthetic medium resulted in a growth defect and it exacerbated the sensitivity to caffeine of this strain. The defects were rescued in plates osmotically stabilized by inclusion of sorbitol (Fig. 3). This suggests that, similarly to *HAL3*, the excess of *VHS3* aggravates the lytic phenotype of the *slt2/mpk1* mutation. However, the effects were less dramatic than those caused by overexpression of *HAL3*. Mutation of *HAL3* has been reported to alleviate *slt2/mpk1* phenotypes, such as caffeine sensitivity. As shown in Fig. 3, a double *mpk1 vhs3* mutant exhibits an increased tolerance to caffeine when compared with the *slt2/mpk1* strain, although *mpk1 vhs3* cells are slightly less tolerant than the *mpk1 hal3* strain.

High copy expression of *HAL3* increases tolerance to high concentrations of sodium and lithium cations. We have found that overexpression of *VHS3* on a wild-type strain also improves salt tolerance (particularly to high sodium), although very slightly. The effect was more dramatic in cells lacking Hal3 (Ref. 18 and Fig. 4), but overexpression of *VHS3* could not increase tolerance in a *hal3 ppz1* mutant (not shown), suggesting that this effect could be mediated by the phosphatase. In all cases, the tolerance conferred by overexpression of *VHS3* was less marked than that obtained by overexpression of *HAL3*. The sensitivity to sodium or lithium of a *vhs3* mutant, assessed in two different genetic backgrounds (JA100 and DBY746), was virtually identical to that of the wild-type strain. It has been reported that overexpression of *HAL3* results in increased expression levels of the *ENA1* Na$^+$-ATPase gene (12). As we show in Fig. 4, high copy expression of *VHS3* in wild-type cells results in a modest increase in expression of a β-galactosidase reporter driven by the entire *ENA1* promoter (about 2.5-fold). This effect is weaker that than obtained by overexpression of *HAL3*. However, it becomes rather intense (about 8-fold) in

cells lacking Hal3. Overexpression of *VHS3* in a *ppz1* background resulted only in a slight increase in the expression driven from the *ENA1* promoter and, similarly to *HAL3*, did not affect expression in a *ppz1 ppz2* strain (Fig. 4).

Vhs3 Binds in Vitro and in Vivo Ppz1—The phenotypes resulting from overexpression or disruption of the *VHS3* gene were consistent with the possibility of Vhs3 acting as a negative regulatory subunit of the Ppz1 phosphatase. To test this possibility we expressed in *Escherichia coli*, as a GST fusion, the catalytic domain of Ppz1 (residues 345–692), which has been shown to bind *in vitro* and *in vivo* Hal3 (11), and used it to construct an affinity system. As shown in Fig. 5, the bacterially expressed catalytic domain of Ppz1 can bind a version of Vhs3 tagged with the FLAG epitope present in yeast cell extracts. In addition, GST-fused versions of the entire Ppz1 and its C-terminal domain were expressed in *S. cerevisiae* from the native *PPZ1* promoter. These cells were transformed with a plasmid bearing the tagged version of Vhs3, extracts were prepared and the Ppz1 derivatives affinity-purified using glutathione beads. As shown in Fig. 5, the purified Ppz1 fractions contained bound Vhs3. The amount of bound Vhs3 was greatly increased when the C-terminal half of Ppz1 was expressed. Previous experiments had shown that the tagged version of Vhs3 was functionally equivalent to the native form of the protein (not shown). These results are consistent with the notion that Vhs3 can interact *in vivo* with the catalytic moiety of Ppz1.

Vhs3 Inhibits in Vitro the Ppz1 Protein Phosphatase Activity—To further test the possibility of Vhs3 being a negative regulatory subunit of Ppz1, we verified the ability of Vhs3 to inhibit Ppz1 *in vitro* by using two different assays. First, we incubated different amounts of GST, GST-Vhs3, or GST-Hal3 with bacterially expressed GST-Ppz1(Δ1–344) and determined the phosphatase activity of the mixtures using *p*-nitrophenyl phosphate as substrate (Fig. 6). We observed that Vhs3 was able to inhibit the phosphatase activity in a dose-dependent fashion almost as effectively as Hal3. The inhibitory effect of Vhs3 was destroyed by heating the protein at 80 °C for 10 min prior to the assay (not shown).

We confirmed the Ppz1 inhibitory capacity of Vhs3 by performing a recently described assay for Ppz1 activity that uses Reg1 as a protein substrate (26). Reg1 is phosphorylated in response to a low glucose signal and, when glucose is added back to the media, Reg1 is dephosphorylated by Glc7 (32). The phosphorylation status of Reg1 can be monitored by changes in SDS-PAGE electrophoretic mobility when expressing an N-terminal domain of the protein. As shown in Fig. 6, Ppz1 readily dephosphorylates Reg1$_{1-443}$ and this dephosphorylation is prevented by previous addition of Vhs3 to the mixture as efficiently as Hal3 does. These results indicate that Vhs3 inhibits Ppz1 activity *in vitro*.

Ppz1 is related in sequence to the catalytic subunit of type 1 protein phosphatases (PP1c), which is represented in yeast by a single gene (*GLC7*). Therefore, we tested the ability of Vhs3 to inhibit Glc7 by using *p*-nitrophenyl phosphate as substrate. Fig. 6 shows that Vhs3 was unable to inhibit PP1c under conditions that allow virtually complete inhibition of Ppz1, while the recently characterized (26) Glc7 inhibitor Ypi1 does.

The hal3 and vhs3 Mutations Are Synthetically Lethal, but Lethality Is Not Caused by Enhanced Ppz Activation—The results presented so far suggested that Hal3 and Vhs3 have somewhat redundant functions as inhibitory subunits of Ppz1. To further test this possibility, we tried to construct a *hal3 vhs3* double mutant by deleting the *vhs3* gene on a haploid *hal3Δ* background. However, none of our attempts were successful. We then transformed a heterozygous diploid *hal3Δ* strain with

FIG. 4. **Effect of *VHS3* overexpression on salt tolerance and *ENA1* promoter activity.** *Upper panel*, wild-type strain DBY746 and its isogenic derivative EDN4 (*hal3*) were transformed with the indicated plasmids. Positive clones were grown in YPD plates supplemented with the indicated concentrations of LiCl or NaCl for 60 h. *Lower panel*, strains DBY746 (*WT*), EDN4 (*hal3*), EDN2 (*ppz1*), and EDN85 (*ppz1 ppz2*) bearing the plasmid pKC201 (which encodes the β-galactosidase reporter gene fused to the *ENA1* promoter) were transformed with the high copy plasmids (YEplac112 or YEplac181) carrying no insert (*empty bars*) or the *HAL3* (*filled bars*) or the *VHS3* (*striped bars*) genes. Cells were grown as indicated under "Experimental Procedures" and β-galactosidase activity measured. Data are presented as the increase in expression compared with the strain carrying the empty plasmid and are mean ± S.E. from three independent clones. *WT*, wild type.
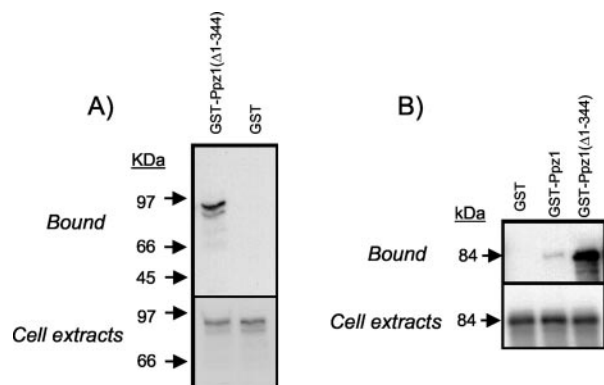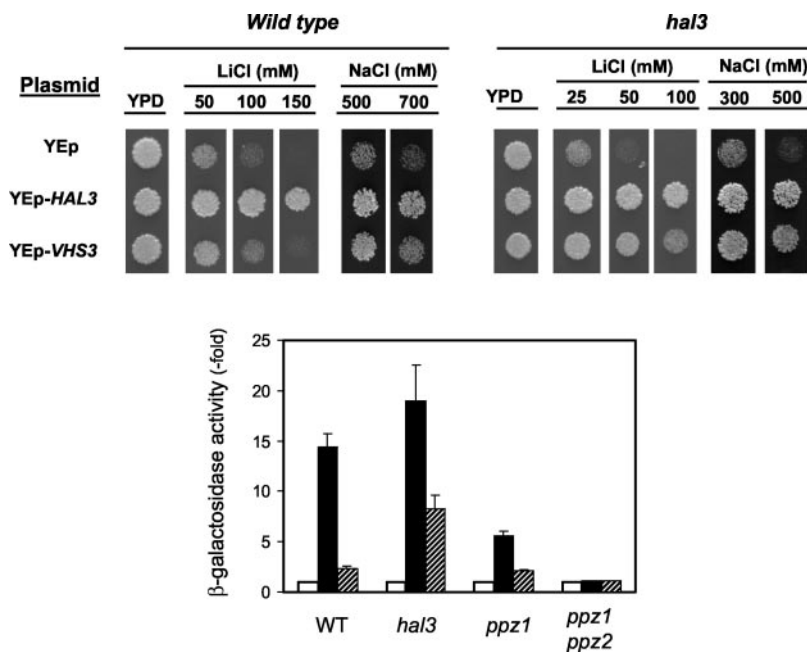

FIG. 5. **Vhs3 binds *in vitro* and *in vivo* to Ppz1.** *A*, cell extracts from strain EDN75 were prepared as described, and 1 mg of total protein was mixed with bacterially expressed recombinant GST or GST-Ppz1(Δ1–344) bound to glutathione-Sepharose beads. The presence of Vhs3–3×FLAG bound to the affinity system was detected using anti-FLAG antibodies. The *lower panel* shows the immunoblot of the cell extracts (20 μg of protein) used in the experiment. *B*, strain EDN75 (*ppz1Δ*) expressing the indicated forms of Ppz1 were transformed with a plasmid expressing Vhs3–3×FLAG. The GST-fused versions of Ppz1 were affinity-purified from total yeast extracts (2.5 mg of total protein) using glutathione-Sepharose beads. The affinity matrix was boiled in 75 μl of SDS-PAGE sample buffer and 15 μl were electrophoresed in 8% SDS-polyacrylamide gels. Proteins were transferred to membranes and Vhs3–3×FLAG detected with anti-FLAG antibodies. The *lower panel* shows the amount of Vhs3–3×FLAG present in the extracts (25 μg of total protein) revealed with anti-FLAG antibodies.

a disruption cassette for *VHS3*, to generate strain MAR6, sporulation was induced and 67 tetrads were dissected. Upon characterization of the resulting spores, we observed 11 non-parental ditypes (with 2 spores giving colonies), 12 parental ditypes (all 4 spores grew), and 44 tetratypes (with only 3 colony-forming spores). Fig. 7*A* shows several samples of the latter possibility. Phenotypic analysis of the growing colonies proved that none of them carried the double *hal3 vhs3* mutation. Microscopic observation of the dissection plates showed that spores unable to produce macroscopic colonies remained as a single cell (not shown). These results suggested that the *vhs3 hal3* mutations might be synthetically lethal. To further test this possibility, we constructed strain MAR107, which carries both *hal3* and *vhs3* mutations and depends on *URA3*-selectable plasmid-borne *HAL3* copies for survival. This strain, as well as

strain MAR88 (*hal3*) carrying the same plasmid, were plated in synthetic medium in the presence of 5-FOA, which does not allow growth of cells carrying the *URA3* marker. While strain MAR88 produced a significant number of colonies in 5-FOA plates, showing that the plasmid could be lost, strain MAR107 did not produce any colony, indicating that the presence of *HAL3* was necessary for vegetative growth in a *hal3 vhs3* background. A similar experiment was carried out with strain MAR115, which depends of the presence of plasmid-borne copies of *VHS3*. Again, plating in the presence of 5-FOA showed that the plasmid could not be evicted. These experiments support the notion that the *vhs3* and *hal3* mutations are synthetically lethal.
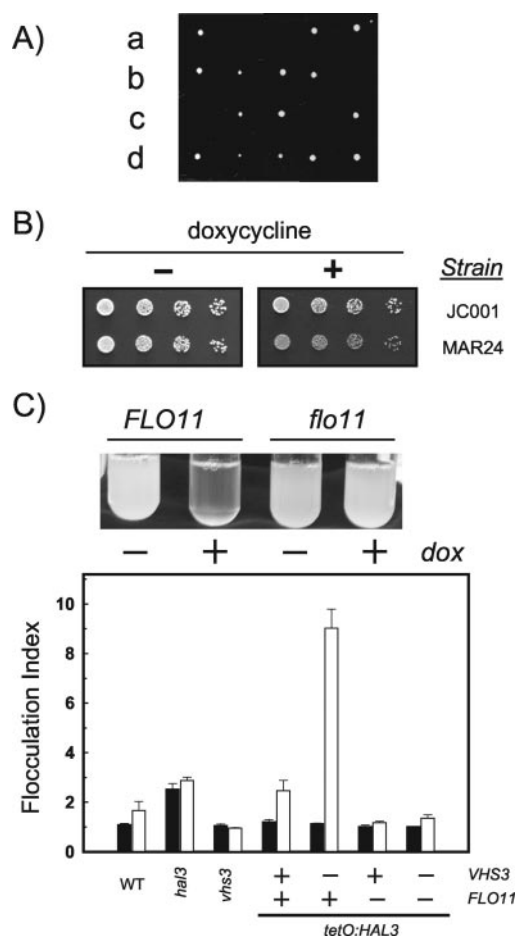
As it is known that an excess of Ppz1 activity could be deleterious for the cell (7) we considered the possibility that the simultaneous absence of *hal3* and *vhs3* could lead to an hyperactive Ppz1, which could explain the synthetically lethal phenotype of this double mutation. To test this possibility, we started from a diploid strain heterozygous for the *hal3* and *vhs3* mutations (MAR6) and introduced in it the *ppz1*, *ppz2,* and *ppz1 ppz2* mutations, respectively, in heterozygosity. These strains were induced to sporulate and tetrads (25 for the triple mutants and 90 for the quadruple one) were dissected. Interestingly, we could not recover double *hal3 vhs3* mutants even in the presence of the *ppz1*, *ppz2* or *ppz1 ppz2* mutations. Furthermore, we transformed the diploid strain MAR112 (heterozygous for the *hal3*, *vhs3*, *ppz1*, and *ppz2* mutations) with plasmid YEp195-HAL3, which provides the *HAL3* gene in a *URA3*-based high copy plasmid. Upon sporulation, haploid transformants carrying all four disruptions and the *URA3* plasmid were recovered and grown on plates containing 5-FOA, to force eviction of the plasmid. We could not recover 5-FOA-resistant colonies, indicating the requirement for the plasmid-borne *HAL3* gene, even in the absence of both phosphatase genes. Identical results were obtained when the *ppz1 ppz2* mutations were introduced in strain MAR107, and eviction of the plasmid was tested. All these results indicate that the deleterious effect of the *hal3 vhs3* mutations was not because of hyperactivation of the Ppz phosphatases.

Effects of the Mutation of His[459] on Vhs3 Functions—His[459] is a conserved Vhs3 residue that also appears in Hal3 and *Arabidopsis thaliana* AtHal3a (Fig. 1). In the plant protein this
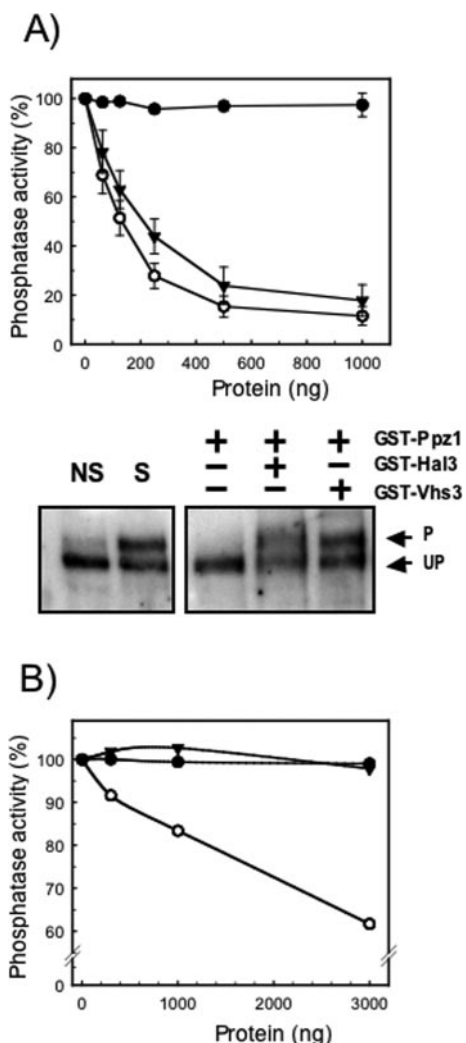
FIG. 6. **Effects of Vhs3 on Ppz1 and Glc7 activities.** *A*, *upper panel*, *p*-nitrophenyl phosphate dephosphorylation assays were carried out as described under "Experimental Procedures." 1 μg of purified GST-Ppz1$_{\Delta1-344}$ was incubated with *p*-nitrophenylphosphate (10 mM) as substrate in the presence of increasing amounts of purified GST (●), GST-Hal3 (○), or GST-Vh3 (▼) proteins. Values are means ± S.E. for three different assays, expressed as percentage of phosphatase activity relative to control without inhibitors. *Lower panel*, HA-Reg1$_{1-443}$ dephosphorylation assays. MCY3000 cells expressing HA-Reg1$_{1-443}$ were grown in high glucose (4%) media and shifted 20 min to low glucose media (0.05%). Crude extracts were then obtained and the phosphorylation state of HA-Reg1$_{1-443}$ before (*NS*, not shifted) or after (*S*, shifted), monitored by SDS-PAGE and immunoblot with anti-HA antibodies. 1.8 μg of purified GST-Ppz1$_{\Delta1-344}$ (GST-Ppz1) were preincubated during 5 min with buffer, GST-Hal3 (2 μg), or GST-Vhs3 (2 μg) and then for 20 min at 30 °C with 1 μg of the extracts. Samples were analyzed by SDS-PAGE (10% polyacrylamide gels) and immunodetected with anti-HA monoclonal antibodies. *P*, phosphorylated; *UP*, unphosphorylated forms of HA-Reg1$_{1-443}$. *B*, 2 μg of purified GST-Glc7 was incubated with *p*-nitrophenyl phosphate in the presence of increasing amounts of purified GST (●), GST-Ypi1 (○), or GST-Vhs3 (▼) proteins. Values correspond to a representative assay, made by duplicate, and are expressed as percentage of phosphatase activity relative to control without inhibitors.

FIG. 7. **Phenotypic effects of the *vhs3 hal3* mutations.** *A*, diploid strain MAR6, heterozygous for the *vhs3* and *hal3* mutations, was induced to sporulate and 67 tetrads were dissected (only five tetratypes are shown). In all cases, spores able to form colonies did not carry the markers associated to both mutations. *B*, strains JC001 (*tetO:HAL3*) and MAR24 (*tetO:HAL3 vhs3*) were spotted at four 1:5 dilutions in synthetic medium in the presence or the absence of 50 μg/ml doxycycline. Growth was monitored after 60 h at 28 °C. *C*, *upper panel*, strains MAR24 (*tetO:HAL3 vhs3 FLO11*) and MAR81 (*tetO:HAL3 vhs3 flo11*) were inoculated in 5 ml of complete minimal medium and grown for 2 days with shaking (230 strokes/min) in the presence or absence of 50 μg/ml doxycycline at 28 °C. Then, tubes were removed from the shaker and photographed immediately. *Lower panel*, indicated strains were grown, as described under "Experimental Procedures" in the presence (*open bars*) or the absence (*closed bars*) of doxycycline and subjected to the flocculation test as described. Signs + and − denote that a given strain carries a wild-type (*WT*) copy or has been deleted for the indicated gene, respectively. The higher the flocculation index, the stronger is the tendency of the strain to flocculate. Data are mean ± S.E. from 3 to 6 experiments.

residue (His[90]) has been postulated to be important for the functional role of the protein (33). To test the relevance of this His in Vhs3, we have mutated His[459] to Ala, and cloned the mutated form, carrying a 3×FLAG C-terminal epitope, in a multicopy plasmid. Diverse phenotypic tests were performed, such as growth in a *slt2/mpk1* background, tolerance to salt in a *hal3* strain, and rescue of the growth defect of a *sit4* strain. In all cases, our results (not shown) indicated that the H495A version behaved identically to the wild-type form. However,

while a wild-type high copy version of *VHS3* did rescue the *hal3 vhs3* lethal phenotype, we could not recover *hal3 vhs3* haploid progeny when the diploid strain MAR6, heterozygous for the *hal3* and *vhs3* mutations, was transformed in either low copy or high copy with the H495A version.

A Conditional vhs3 tetO:HAL3 Double Mutant Displays a Flocculation Phenotype and Has Increased FLO11 Expression—The results described above were compatible with the possibility of Vhs3 (and also Hal3) having unsuspected functions, perhaps unrelated to its role as regulator of the Ppz1 protein phosphatase. To gain insight into which could be these functions we constructed a conditional *hal3 vhs3* double mutant (strain MAR24) by deleting the *VHS3* gene and replacing the *HAL3* promoter by a doxycycline-regulatable *tetO* promoter. When this strain was plated in the presence of 20–100
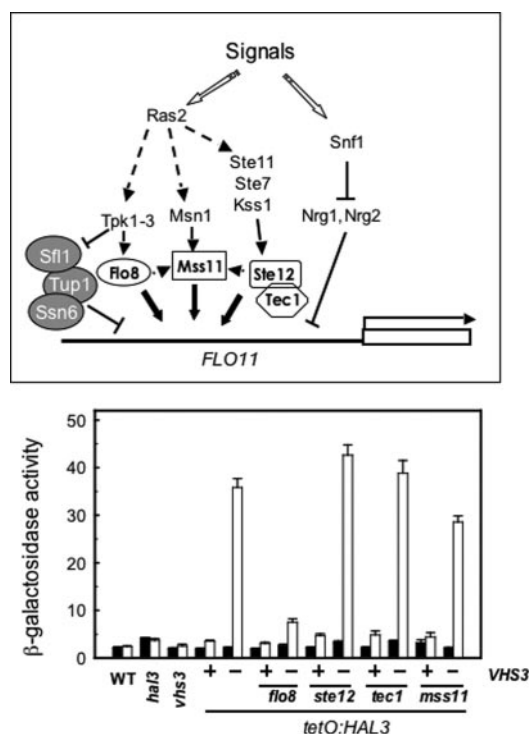
FIG. 8. *FLO11 expression in different mutant backgrounds.* *Upper panel*, major signaling pathways regulating *FLO11* expression. This schematic depiction integrates information taken from Refs. 52–57, omitting multiple elements of the system for the sake of clarity. *Lower panel*, indicated strains were transformed with plasmid pFLO11-LacZ, and cultures were grown for 15 h at 28 °C in YPD medium as indicated in the presence (*open bars*) or the absence (*closed bars*) of 100 μg/ml doxycycline. Cells were then collected and processed for β-galactosidase activity assay. Data are mean ± S.E. from 6 to 12 independent transformants. *WT*, wild type.

μg/ml doxycycline, growth was somewhat reduced, but cells were still able to proliferate (Fig. 7*B*). Interestingly, when cultured in liquid medium, the presence of doxycycline resulted in a readily detectable flocculation phenotype (Fig. 7*C*). However, flocculation was not produced when the same strain was deleted for the *FLO11* gene, encoding a glycosylphosphatidyl-inositol-anchored cell surface protein required for cell-cell adhesion. Quantitative analyses were performed to monitor more accurately the flocculation phenotype. As shown in Fig. 7*C* (*lower panel*), a *vhs3* mutant does not flocculate, whereas a *hal3* mutant shows a weak tendency to flocculate, similar to that of strain JC001 (*tetO:HAL3*) in the presence of doxycycline. Flocculation was greatly enhanced in strain MAR24 when grown in the presence of the antibiotic, and this effect was fully abolished when the *FLO11* gene was deleted, indicating that the flocculation phenotype resulting from the lack of Hal3 and Vhs3 function requires the Flo11 flocculin.

Because the control of *FLO11* expression is an important way to regulate its function (see "Discussion"), we considered interesting to test whether strain MAR24 could have an increased *FLO11* expression under non-permissive conditions. To this end, a β-galactosidase reporter containing the entire *FLO11* promoter was introduced in different strains and the activity of the promoter determined in the presence and absence of doxycycline. As it can be observed (Fig. 8), addition of doxycycline to strain MAR24 results in about 15-fold increase in *FLO11* expression. Interestingly, this effect was largely abolished when this strain was deleted for *FLO8*, a gene required for *FLO11* induction following protein kinase A activation. In contrast, deletion of *STE12*, *TEC1*, or *MSS11*, encoding elements that mediate other activating pathways (see Fig. 8 and

"Discussion") did not affect *FLO11* expression under the conditions tested.

DISCUSSION

The gene *VHS3* (YOR054c) was identified in a recent screen in search of multicopy genes able to suppress the G_1/S blockade of a conditional *sit4 hal3* mutant. This gene encodes a 674-residues protein that is closely related to *S. cerevisiae* Hal3 (49% of identity) and also contains a characteristic C-terminal highly acidic tail that, in the case of Hal3, has been shown to be essential for function in salt tolerance and cell cycle (12, 13). These features and the fact that there was essentially no functional information available for *VHS3* prompted us to characterize in detail this gene and to investigate whether it could be a functional homologue of Hal3. We have observed that overexpression or deletion of *VHS3* results in a number of phenotypes that are reminiscent to those detected in cells that overexpress or lack *HAL3*. As we demonstrated earlier for Hal3 (11), Vhs3 binds *in vivo* to the catalytic moiety of Ppz1 more efficiently than that to the entire protein, and it can inhibit *in vitro* the activity of the phosphatase almost as efficiently as Hal3 does. Therefore, Vhs3 can be considered as an inhibitory subunit of Ppz1. We have also observed that, for all these phenotypes, the effects of the overexpression or the absence of Vhs3 are less dramatic that those observed for Hal3, suggesting a less prominent biological role. This could have several explanations. For instance, Vhs3 is almost fully recovered in $16,000 \times g$ supernatants, indicating that is a soluble protein (not shown). In contrast, a substantial amount of Ppz1 (7) and Hal3 (data not shown) is recovered in particulate fractions. Therefore, despite that Vhs3 can effectively inhibit Ppz1, only a limited fraction of the phosphatase might be able to interact with Vhs3 *in vivo*. Alternatively, a difference in their expression levels could account for the intensity of the observed phenotypes. Although the comparison has not been carried out at the protein level, Northern blot and DNA microarray data would support this possibility because, at least in exponentially growing cells, *VHS3* mRNA levels are almost undetectable and clearly lower than those of *HAL3*.[2]

Overexpression of Ppz1 negatively affects cell growth and leads to a G_1 blockage that can be alleviated by high levels of Hal3 (8). We hypothesized that the synthetically lethal phenotype of the *hal3 vhs3* mutation could be due to an abnormally increased Ppz1 activity caused by the absence of its natural inhibitors. However, we have observed that the lethal phenotype is maintained even in the absence of both *PPZ1* and *PPZ2* phosphatase genes. This is in contrast with the situation defined by the synthetic lethality of the *sit4* and *hal3* mutations, which is rescued by deletion of *PPZ1* (8). In addition, current work in our laboratory has revealed a number of mutations in the Hal3 protein that abolishes binding and/or inhibition of Ppz1. However, none of these mutations affect the ability of Hal3 to rescue growth of a *hal3 vhs3* strain.[3] Therefore, these results do not support our initial hypothesis and suggest for the first time that Vhs3 or/and Hal3 must have important, Ppz-independent functions. It has been reported that Ppz1 and Ppz2 may have overlapping functions with Glc7, the essential type 1 protein phosphatase in yeast, and that all three phosphatases may act through common regulatory proteins (34, 35). Because high levels of Glc7 activity are lethal (36–38) an alternative possibility to explain the synthetic lethal phenotype of the *hal3 vhs3* mutation would be that these proteins could be inhibiting Glc7. However, it has been proved that Hal3 does not

interact with Glc7 nor inhibit its activity (11, 26), and we show here that Vhs3 cannot inhibit Glc7 *in vitro*. In addition, mutation or overexpression of *VHS3* did not affect glycogen accumulation (not shown), a characteristic phenotype attributed to modulation of Glc7 function (26, 34, 39). Therefore, it is not likely that the lethal phenotype of the double *hal3 vhs3* mutant could be attributed to Glc7 hyperactivation.

The plant *A. thaliana* encodes a protein similar to Hal3 and Vhs3, named AtHal3a (Fig. 1), which has been characterized in some detail. Expression of this flavoprotein in *hal3* yeast cells partially complements the characteristic LiCl sensitivity of this strain, and transgenic *Arabidopsis* plants that overexpress AtHal3a show improved tolerance to salt stress (40). The elucidation of its crystal structure (33) allowed the prediction of catalytic properties to AtHal3a, and it has been shown that the recombinant protein has the capacity to catalyze *in vitro* the decarboxylation of 4′-phosphopantothenoylcysteine to 4′-phosphopantetheine (41, 42), one of the steps in coenzyme A biosynthesis. This catalytic capacity involves AtHal3a His[90], as mutation of this residue to Ala leads to the complete inactivation of the enzyme. We have observed that the H459A change in Vhs3 produces a functional protein, as far as the Ppz-related phenotypes are concerned. However, this same change produced a protein unable to rescue the synthetically lethal phenotype of the *hal3 vhs3* mutant, supporting again the notion that this phenotype is unrelated to Ppz functions. Although this could lead to speculation that the lethal phenotype may be the result of loss of the above catalytic activity, it must be noted that AtHal3a also requires a conserved Cys[175] within the substrate recognition clamp to complete the catalytic reaction (41, 43). However, both Hal3 and Vhs3 lack the equivalent residue, which corresponds to Asn in both proteins. Therefore, the lethal effect of the absence of Hal3 and Vhs3 is not likely to be the result of a defect in this hypothetical catalytic activity.

Our tetrad analysis experiments showed that the spores that failed to form colonies remained as single cells. This is interesting as often maternal cells provide enough products to carry out al least a few cell divisions. This might reflect that the lack of Vhs3 and Hal3 activities could result in impossibility to germinate, rather than in blockage of vegetative cell growth. However, while formally a role of *hal3* and *vhs3* in germination cannot be discarded, we also show that vegetatively growing *hal3 vhs3* cells that survive because the presence of episomal copies of *HAL3* or *VHS3* cannot lose the plasmids, indicating that simultaneous lack of *hal3* and *vhs3* functions is incompatible with vegetative growth.

In an attempt to gain insight into the novel functions of Hal3 and Vhs3 we have constructed a conditional *tetO:HAL3 vhs3* mutant, which allows the turning off of the expression of *HAL3* when cells are grown in the presence of doxycycline. This approach has been successfully used in the past in our laboratory to explore the nature of the synthetically lethal phenotype of the *hal3 sit4* mutations (17, 18). Interestingly, and in contrast to the phenotype observed in the *tetO:HAL3 sit4* strain, the presence of doxycycline did not result in severe growth arrest, but in a flocculation phenotype. The observation that a conditional double mutant based in the *tetO*-regulatable expression of *HAL3* fails to reproduce the *vhs3 hal3* growth defect may appear in conflict with our observations described above. However, similar situations have been widely documented for a substantial number of essential genes (44–46), and could be explained by a residual expression from the *tetO:HAL3* construct. In any case, analysis of the conditional mutant strain MAR24 has led us to the identification of a novel phenotype associated to decreased expression of Hal3 and Vhs3: the ability to flocculate. It is known that a critical factor for flocculation

of yeast cells is the presence or the absence of lectin-like proteins (flocculins), such as Flo1, Flo5, and Flo9, the so called *FLO1*-type phenotypic class, and Flo11. These proteins are present in the cell walls of flocculent cells and allow selective binding of mannose residues located in the cell walls of adjacent yeast cells (see Ref. 47 and references therein). The possibility that Flo11 might be involved in the flocculation phenotype of the conditional *hal3 vhs3* mutant draws our immediate attention, because this flocculin has been shown to be involved in diploid pseudohyphal differentiation (48–50) and haploid invasive growth (48, 49, 51) in response to nutrient limitation. We demonstrate here that the flocculation phenotype due to the absence of Vhs3 and Hal3 function is dependent on the presence of Flo11 and that is accompanied by an increased expression from the *FLO11* promoter, suggesting that Vhs3 and Hal3 are negative regulators of *FLO11* expression.

FLO11 has one of the largest promoters in the yeast *S. cerevisiae*. This promoter integrates multiple signals mediated by at least 4 major pathways (see Fig. 8 for a diagram and Refs. 52–57). Protein kinase A activation inhibits the repressor complex Sfl1-Tup1-Ssn6 and activates the positive transcription factor Flo8. In addition, the invasive growth MAP kinase cascade results in the activation of the MAP kinase Kss1, which in turn activates the Ste12/Tec1 transcription factor system. The Mss11 transcription factor, which shows a limited homology with Flo8, is also a positive effector of *FLO11* transcription. All three pathways can be activated by the small guanine nucleotide-binding protein Ras2. In addition, a recently discovered pathway involves the AMP-activated kinase yeast homolog Snf1 in the regulation of *FLO11* expression by means of the Nrg1/Nrg2 repressors. We have observed that the increased expression from the *FLO11* promoter in strain MAR24 is almost fully blocked in the absence of Flo8, while deletion of the *STE12*, *MSS11*, *TEC1*, or *STE12* genes did not affect the activity of the promoter (Fig. 8). In addition, deletion of *FLO8* abolished the flocculent phenotype, while the *SNF1* mutation did not (not shown). These data are consistent with the notion that Vhs3 and Hal3 probably function upstream of the Flo8 transcription factor. Although further analysis will be required to elucidate the exact role of these proteins on the regulation of Flo8, an important point within the context of this work would be to evaluate whether or not this function is mediated by the Ppz phosphatases. Our preliminary data suggest that Ppz1 is probably not a key component of the system because, although cells overexpressing the phosphatase display some tendency to flocculate and have slightly increased *FLO11* expression, these effects are much weaker than those produced by the absence of Vhs3 and Hal3. In addition, low copy expression of the H459A version of Vhs3 completely fails to abolish flocculation of strain MAR24 in the presence of doxycycline (data not shown).

In conclusion, we have demonstrated that the previously uncharacterized protein Vhs3 can act as a negative regulatory subunit of Ppz1, thus, adding an additional level of complexity to the regulation and function of this protein phosphatase. In addition, we also show here that Vhs3 (and perhaps Hal3) fulfill essential functions that are independent of their role in regulating Ppz1. The existence of proteins able to carry out different and unrelated cellular functions (known as moonlighting proteins) has been reported in the last few years (see Refs. 58 and 59 for reviews). Perhaps Hal3 and Vhs3 are new examples of these kinds of proteins.

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