Chapter 6

Identification of Constitutively Active AtMPK6 Mutants Using a Functional Screen in *Saccharomyces cerevisiae*

Elodie Hudik, Souha Berriri, Heribert Hirt, and Jean Colcombet

Abstract

MAPK (Mitogen-Activated Protein Kinases) mutants which are active independently of phosphorylation by upstream MAPK Kinases (MAPKKs) help to clarify signal transduction processes through MAPK modules and provide a useful tool to understand MAPK roles in the cell. The identification of such mutations is tricky. In this chapter, we provide a detailed protocol for their screening, taking advantage of a functional expression assay in yeast.

Key words MAP Kinase, Functional genetic screen, Constitutive activity, *Saccharomyces cerevisiae*, In vivo cloning, Random mutagenesis

1 Introduction

Constitutively active (CA) protein kinases considerably extend the repertoire of tools for the functional analysis of signal transduction pathways [1]. Until recently, no strategy to render MAPKs (Mitogen-Activated Protein Kinases) was available for plants. In a recent work, we took advantage of a functional expression screen in yeast to identify point mutations triggering MAP2K-independent constitutive activity to a model MAPK of *Arabidopsis*, MPK6 [2]. In this chapter, we provide a detailed protocol for the screening of CA MAPKs in yeast.

The *Saccharomyces cerevisiae* genome codes for four functional MAPK modules [3]. The high osmolarity glycerol (hog) pathway, defined by the MAPKK pbs2 and the MAPK hog1, is activated in response to high osmolarity and allows the yeast to survive with large concentrations of salt or solutes in the environment (Fig. 1). Single and double mutants of the pathway are hypersensitive to salt and will die on high salt concentrations. In a previous work, we showed that a number of MAP2K/MAPK modules, including *Arabidopsis* AtMKK2/AtMPK6, are able to functionally complement the defect of growth of *pbs2Δhog1Δ* double mutants [4].

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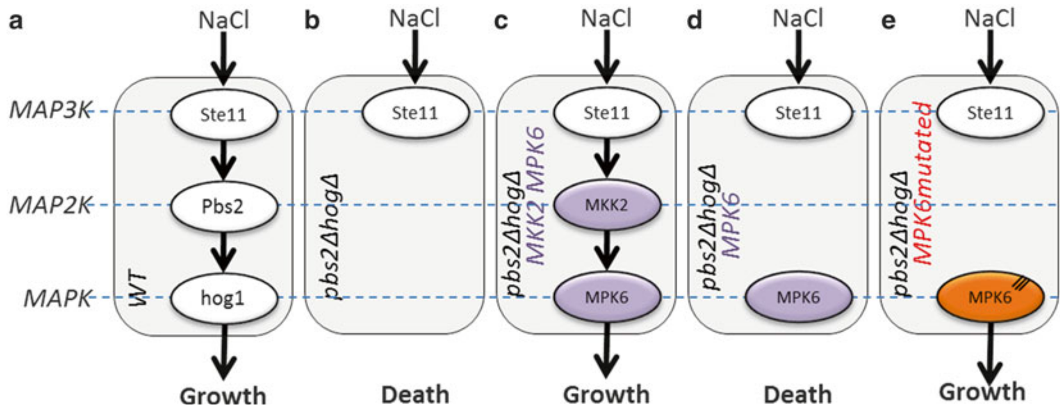

Fig. 1 Overview of the screening strategy using a mutant yeast strain defective in hog1 pathways. In *Saccharomyces cerevisiae*, NaCl activates the high-osmolarity glycerol 1 (Hog1) MAPK module, allowing the cells to grow on media supplemented with salt (**a**). *pbs2*Δ *hog*Δ double-mutant is unable to cope with high salt concentrations (**b**), but this growth defect is complemented by *Arabidopsis thaliana MPK6* and *MKK2* genes (**c**). The fact that *MPK6* alone does not complement the defect suggests that MKK2 is necessary for MPK6 activation (**d**). The functional screen consists to identify MPK6 mutations triggering the yeast growth on salt media without its upstream MAP2K MKK2 (**e**)

Interestingly, MKK2 activation of MPK6 is necessary for growth recovery, as MPK6 alone is unable to restore growth on hyperosmotic conditions. This defines a suitable screening condition to identify mutations which allow MAP2K-independent MAPK activity as mutations in MPK6 triggering improved catalytic activity should complement the growth defect phenotype of the yeast double mutant on salt (Fig. 1).

hog1-related mutants are able to grow on normal synthetic media, but not under hyperosmotic conditions, allowing for an easy selection system in the laboratory. Additionally, the CA MAPK screen using such strains is a positive screen: the interesting clones will be the ones able to grow on a selective hyperosmotic medium. The protocol we describe here is largely adapted of the work of Englberg and coworkers [5]. The major modification we performed consists in generating mutations using a PCR-based random mutagenesis strategy coupled to an in vitro cloning step instead of using an *E. coli* strain inducing mutations to amplify the plasmids. Figure 2 shows an overview of the molecular cloning steps allowing the creation of a library of randomly mutated MPK6 open reading frames (ORFs) ready to be expressed in the reporter yeast strain (e.g., Fig. 2). With this technique (we used GeneMorph® II Random Mutagenesis Kit from Stratagene), the number of mutations is accurately selected ranging from 0–4.5 to 9–16 mutations per kb depending on the PCR conditions. The Mutazyme II polymerase activity of the kit is a mixture of several Taq DNA Polymerase enzymes performing different kinds of errors during

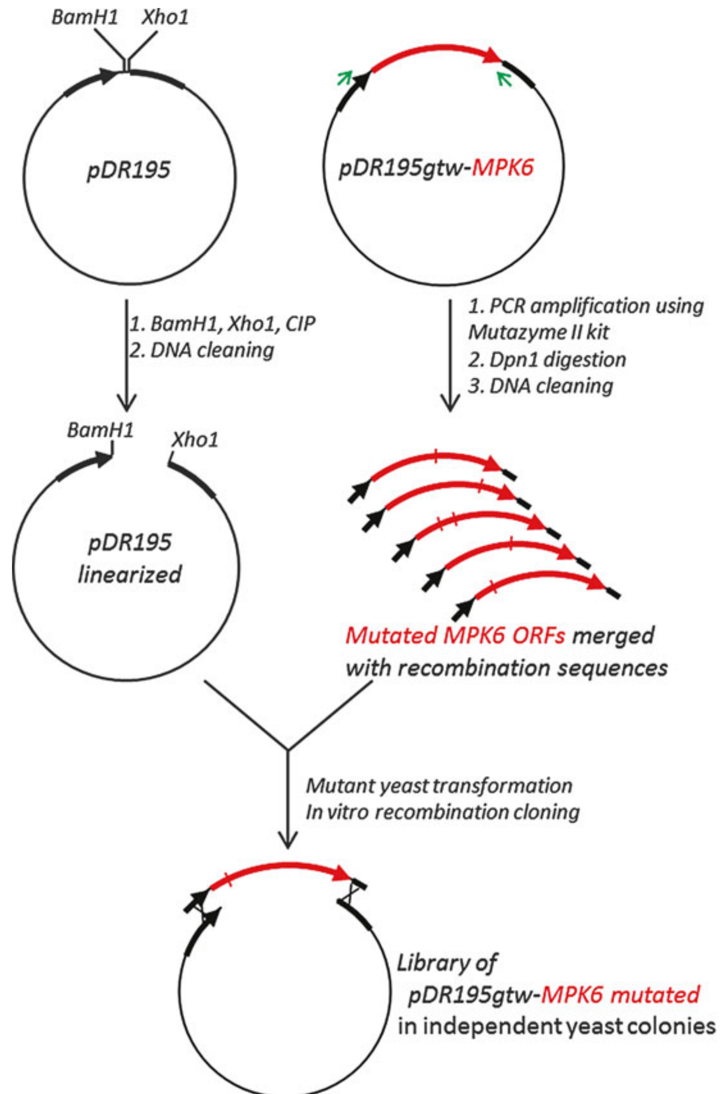

Fig. 2 Reconstruction in yeast of a plasmid library allowing for the expression of randomly mutated MPK6

the DNA synthesis in a way to have similar probabilities to obtain all types of mutations. Please note however that each randomly created mutation has about a 5 % chance to induce a STOP codon. Therefore, there is a risk to lose a large number of clones which are truncated by using a high mutation rate protocol on long sequences.

2 Materials

A list of materials is provided as example, but similar materials from other brands may be purchased.

2.1 Strains and DNA

1. *Saccharomyces cerevisiae pbs2Δhog1Δ* double mutant in the genetic background W303 [4, 6].
2. DH5alpha thermo-competent *Escherichia coli* cells prepared as previously described [7].
3. pDR195 and pDR195gtw-MPK6 (6, 1). pDR195-based vectors contain the URA3 autotrophy marker gene. Other expression vectors and MAPK model gene may be used to perform a screen as long as they allow the complementation of the strain defect as described in Fig. 1. This will allow for the identification of new mutations.
4. Primers pPMA1prom (TTT CTC TTT CTT TCC TAT AAC ACC AAT AGT G) and pADH1term (GTG TCA ACA ACG TAT CTA CCA ACG ATT TGA CC) for MPK6 amplification from pDR195gtw-MPK6.
5. Primers pMPK6_F (GTA TTT TCT TTA CCA GAT CCT CCG TG) and pMPK6_R (CTG GTG CAC GGT ACC ATC TCG TGA C) for the sequencing of the MPK6 coding sequence.

2.2 Enzymes and Kits

1. Endonucleases BamH1 and Xho1, calf intestinal alkaline phosphatase, and NEBuffer3 were all from NEB.
2. Wizard® SV Gel and PCR Clean-Up System (Promega).
3. Wizard® SV plus Minipreps DNA Purification System (Promega).
4. GeneMorph® II Random Mutagenesis Kit (Stratagene).

2.3 Medium and Buffer

1. YPD medium: (1 % (w/v) Yeast extract, 2 % (w/v) peptone, 2 % (w/v) d-glucose, ±2 % (w/v) Agar for plates, autoclaved).
2. SC-U (Synthetic medium minus Uracil) medium: (0.17 % (w/v) Yeast Nitrogen Base w/o amino acids and ammonium sulfate, 0.5 % (w/v) NH_4SO_4, 2 % (w/v) d-Glucose, Drop-out mix minus Uracil (US Biological), pH 5.6 adjusted with NaOH, ± 2 % (w/v) Agar for plates, autoclaved).
3. LB (Lysogeny Broth) medium and LB agar.
4. TE Buffer: 10 mM Tris–HCl pH 7.5, 1 mM EDTA–NaOH pH 8.0.
5. TEL0.5 Buffer: (0.5 mM EDTA–NaOH pH 8.0, 5 mM Tris–HCl pH 7.5, 100 mM lithium acetate pH 7.5, filtered).
6. TEL5 Buffer: 5 mM EDTA–NaOH pH 8.0, 50 mM Tris–HCl pH 7.5, 500 mM lithium acetate pH 7.5, filtered.
7. PEG solution: 50 % (w/v) PEG-3350 in water, filtered.
8. PegTEL Buffer: 1 volume of TEL5 Buffer + 4 volumes PEG solution.
9. Yeastmaker Carrier DNA (Clonetech).

10. Yeast Extraction Buffer: 100 mM NaCl, 10 mM Tris–HCl pH 8, 1 mM EDTA, 0.1 % (w/v) SDS.

2.4 Specific Materials

1. Nanodrop 2000c (Thermo Scientific).
2. Replicator (Replica-Plating Tool, Scienceware) with adapted autoclaved cotton velveteen squares.

2.5 Consumables

1. Glass beads, acid washed (e.g., Sigma).
2. Plating beads (autoclaved).
3. Ø 14.5 cm round petri dish.
4. Ø 8 cm round petri dish.
5. 96-wells plates with 2 mL square wells for microbiological culture.
6. Gas-Permeable Adhesive Seals (e.g., Thermo Scientific).

3 Methods

The pipeline of the whole process is presented in Fig. 3.

3.1 Generation of DNA Mixture for *pbs2Δhog1Δ* Yeast Transformation

1. Prepare mutagenized PCR product using GeneMorph® II Random Mutagenesis Kit (Stratagene), pPMA1prom and pADH1term as primers and pDR195gtw-MPK6 as matrix. pPMA1prom and pADH1term couple of primers amplifies a

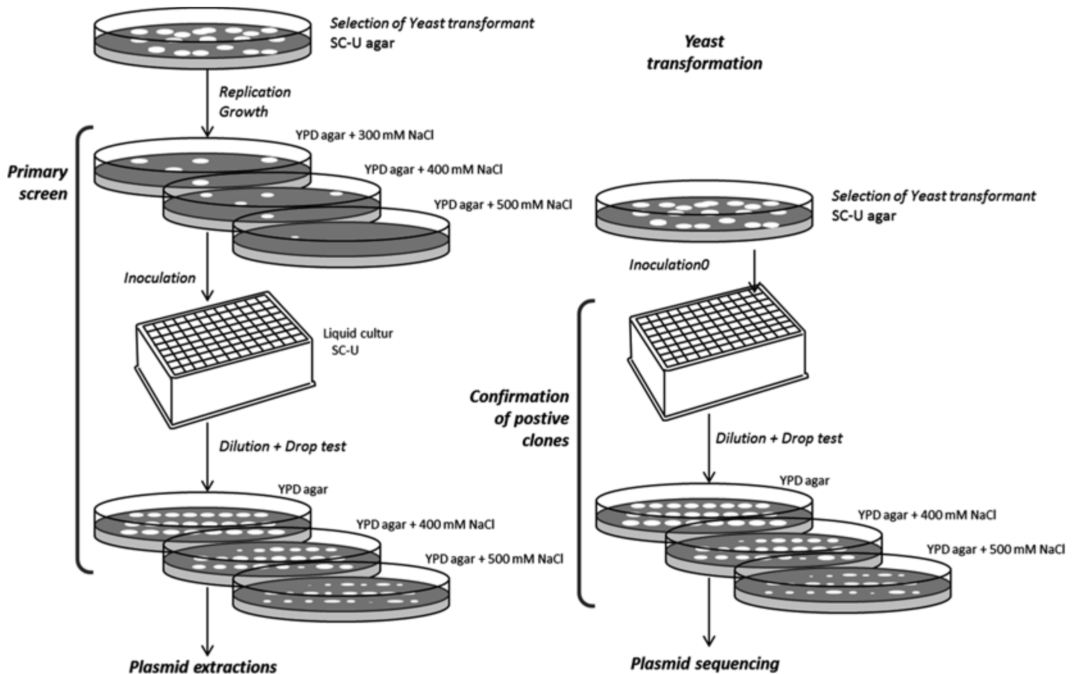

Fig. 3 Overview of the protocol

1,621-bp DNA fragment from pDR195gtw-MPK6. We generally perform three 50 μL PCR reactions in parallel in order to rich different mutation rates as stated by the manufacturer (Low: 0–4.5, Medium: 4.5–9, and high: 9–16 mutations/kilobases).

2. After the amplification reaction, digest the matrix by adding 2 μL Dpn1. Mix well by pipetting, spin down, and incubate at 37 °C for 2 h.
3. Clean the PCR DNA using a micro-column. We commonly use Wizard® SV Gel and PCR Clean-Up System (Promega).
4. Measure the DNA concentration using Nanodrop.
5. Meanwhile, linearize 5 μg of pDR195 with BamH1 and Xho1 in the presence of CIP by mixing:

DNA	5 μg
NEB buffer 3 (10×)	5 μL
BamH1	2 μL
Xho1	2 μL
CIP	0.5 μL
H_2O	up to 50 μL

6. Spin down the reaction mix at maximum speed for 10 s and incubate 2–4 h at 37 °C.
7. Clean the DNA using a micro-column. We commonly use Wizard® SV Gel and PCR Clean-Up System (Promega).
8. Measure the DNA concentration using a Nanodrop or another adapted device.
9. Prepare in 1.5 mL tubes, DNA mixtures with the linearized vector and the PCR product with a 1:1 ratio in molarity. Prepare controls as followed. Keep on ice for transformation.

3.2 Transformation of *pbs2Δ hog1Δ* Yeast

1. Inoculate an YPD agar plate with the glycerol stock of *pbs2Δ hog1Δ* yeast using a sterile inoculator loop. Grow the yeast 2–3 days at 30 °C.
2. Inoculate 5 mL YPD in 50 mL culture tube with a single colony and grow the culture at 30 °C overnight (ON) at 180 RPM.
3. Dilute the 5 mL ON culture in 50 mL YPD in 200 mL Erlen Flask and grow for an additional 5–6 h at 30 °C with 180 RPM.
4. Spin down the cells at 2500 RPM (~1,300 × *g*) at 20 °C for 5 min.
5. Resuspend by pipetting the pellet in few milliliters of TE buffer. Complete to 50 mL with TE buffer.

Table 1
Overview of the DNA mixes to be prepared for the yeast transformation

DNA mix	Number of tubes to be prepared	Meaning	Expected number of expected colonies
Water::water	1	Test of the contamination of the solutions used in the yeast transformation protocol	None
Linearized vectors::water	1	Check the linearization and the ability of the yeast to repair DNA	Few (ideally none)
Water::Mutated PCR fragment with a low mutation level	1	Check the Dpn1 degradation of the matrix pDR195-MPK6 used for the mutagenesis	Few (ideally none)
Water::Mutated PCR fragment with a medium mutation level	1	Check the Dpn1 degradation of the matrix pDR195-MPK6 used for the mutagenesis	Few (ideally none)
Water::Mutated PCR fragment with a high mutation level	1	Check the Dpn1 degradation of the matrix pDR195-MPK6 used for the mutagenesis	Few (ideally none)
Linearized vectors + Mutated PCR fragment at low level	5[a]	Putative interesting clones	Many
Linearized vectors + Mutated PCR fragment at medium level	5[a]	Putative interesting clones	Many
Linearized vectors + Mutated PCR fragment at high level	5[a]	Putative interesting clones	Many
pDR195 + MPK6 (1 μg)	1	Quantification of the yeast transformation efficiency	Many

[a]The total number of transformation tubes is about 20 corresponding to the use of 2 mL of yeast competent cells (*see* below)

6. Spin down the cells at 2500 RPM (~1,300 × *g*) at 20 °C for 5 min.
7. Resuspend the pellet in 2 mL TEL0.5 buffer.
8. Keep the pellet for 10 min at room temperature (and longer on ice).
9. Meanwhile mix each transformation DNA mixture (*see* Table 1) with 10 μL denaturated carrier DNA. Process controls at the same time (*see* Table 1). Keep them on ice up to next step.
10. Add 100 μL of competent yeast. Mix well by pipetting.
11. Add 700 μL of PegTEL Buffer. Mix well by vortexing.
12. Incubate at 30 °C for 30 min with slow shaking.
13. Add 88 μL DMSO and vortex.

14. Heat-shock the cells in a water-bath at 42 °C for 7 min.
15. Spin down the cells 1 min at 5,000 RPM (~2,700×*g*) in a microtube centrifuge.
16. Remove the supernatant and resuspend the cells in 1 mL TE Buffer.
17. Spin down the cells 1 min at 5,000 RPM (~2,700×*g*) in a microtube centrifuge.
18. Remove supernatant using a 1 mL pipette and resuspend the cells in 200 μL sterile TE Buffer.
19. Pour the appropriate number (20) of SC-U agar plates (usually before the experiment or during the 30 min incubation at 37 °C). Let them solidify and dry without lid up to the point the agar surface presents wavelets.
20. Place 5–30 plating glass beads on each LB agar plates.
21. Pipette the transformed yeast cells (~200 μL) on the medium surface.
22. Spread the yeast solution at the agar surface using horizontal shaking long enough for the liquid to be sucked by the agar medium.
23. Invert the plates to collect the glass beads in the cover. Throw them in a collector to be later on cleaned and reused.
24. Let the yeast colonies grow during 2–3 days at 30 °C.
25. Roughly evaluate the number of transformant colonies (*see* **Note 1**).

3.3 Identification of Yeast Colonies Able to Grow on Salt

1. Prepare YPD agar selective plates containing salt by mixing 25 mL of melted YPD agar medium with an appropriate volume of sterile 5 M NaCl. We usually prepare for each plate of primary transformant colonies two series of five plates with various concentrations of NaCl (350—400—450—500—550 mM). Label them with the Salt concentration, the order they will have in the replication process and a reference to the first SC-U agar plate containing the primary transformant colonies.
2. Sterilize the replicator using 70 % (v/v) ethanol. Velvet squares should have been autoclaved.
3. Place cotton velveteen square on the replicator and clamp it using the adapted locking ring.
4. Orientate the SC-U agar plate containing the colonies, the 10 salt YPD plates, and the replicator by drawing a dot on the bottom of the plates and on the ring of the replicator.
5. Apply medium surface of the SC-U agar plate containing the primary colonies on the replicator velvet, marrying the two orientation dots up. Press gently for yeast cells to be transferred onto the velvet. Remove the plate.

6. Apply one by one the 10 YPD agar plates containing Salt, marrying the orientation dots up. Each time, check carefully that the velvet tissue is in contact with the agar medium by gentle tapping fingers.
7. Incubate the plates long enough to see colonies appearing (*see* **Note 2**).
8. Strip candidate colonies on a SC-U plate to back them up long enough to run the experiment to the end.

3.4 First Confirmation of Yeast Candidates

1. Fill a 96-well plate with 500 μL SC-U liquid medium.
2. Inoculate using sterile toothpicks SC-U medium with individual candidate yeast colonies. Include also the strain transformed with pDR195gtw-MPK6 as negative control.
3. Seal the plate with gas-permeable adhesive seal.
4. Grow the yeast overnight at 30 °C under constant shaking.
5. The morning after, pour large petri dishes with 75 mL YPD supplemented with a range of salt concentrations as defined above by mixing melted YPD agar with an appropriate volume of sterile 5 M NaCl. Let solidify and dry up.
6. Fill a 96-well PCR plate (dilution plate) with 200 μM of TE.
7. As yeast cells tend to sediment, resuspend the overnight yeast culture by pipetting. Faster work can be performed using a 300 μL multichannel pipette.
8. Dilute 10 μL of each yeast culture in 200 μL TE. Mix well.
9. Use a multichannel pipette to drop 5 μL of each diluted yeast candidate clone on medium.
10. Let the yeast drop dry.
11. Incubate the plates at 30 °C for 2–4 days.

3.5 Recovery of Plasmids from Candidate Yeast Colonies

1. Inoculate with the confirmed yeast strains recovered from the selective salt YPD agar, 5 mL SC-U liquid culture in 50 mL culture tubes. Incubate overnight at 30 °C with shaking.
2. Fill a 2 mL Round bottom microfuge tube and spin down the cells from 2 mL of culture (5,000 RPM (~2,700 × *g*) for 1 min).
3. Decant the supernatant by inverting the tubes.
4. Add about 300 μL of acid-wash glass beads.
5. Add 300 μL of Yeast Extraction Buffer.
6. Vortex 10 times during 30 s and keep the tubes on ice during 30 s in between.
7. Add 500 μL Phenol. Mix well.
8. Centrifuge at maximum speed for 5 min.
9. Transfer the supernatant (about 400 μL) in a new clean 2 mL tube. Add 1/10 volume Acetate Na 3 M and 3 volumes of

absolute cold ethanol. Mix well and keep in freezer for at least 30 min.

10. Centrifuge at 14,000 RPM (~20,000 × *g*) for 10 min.
11. Drain out the supernatant and wash the pellet with ice-cold ethanol 70 % (v/v) for 1 min.
12. Centrifuge at 14,000 RPM (~20,000 × *g*) for 2 min.
13. Rapidly remove the supernatant with a 1 mL pipette and briefly spin down to drain the remaining liquid and remove it by pipetting.
14. Let the pellet dry at room temperature.
15. Dissolve the pellet in 30 μL TE. Warm to 40 °C for 5 min to help dilution if necessary.
16. Transform 5 μL of the yeast extract in DH5 alpha thermo-competent *E. coli* cells. Plate the bacteria on LB agar plate supplemented with 100 ng/mL Carbenicillin (*see* **Note 3**).
17. Incubate at 37 °C overnight.
18. Select a single colony for each candidate to start 5 mL LB liquid culture in 50 mL tubes. Grow them overnight at 37 °C under constant shaking.
19. Extract the plasmid. We commonly use Wizard® SV plus Minipreps DNA Purification System (Promega).

3.6 Confirmation that the Mutations of the Candidate Plasmids Confer Salt Tolerance to *pbs2Δhog1Δ* Yeast

1. Using the yeast transformation protocol described above, transform 5 μL DNA of each candidate plasmid in the yeast mutant *pbs2Δhog1Δ*. Add pDR195 and pDR195gtw-MPK6 plasmids as negative controls. Do not forget a technical negative control as well (transformation with water) for which not a single yeast colony should be recovered. Plate the transformed yeast on SC-U in 8 cm plates (*see* **Note 4**). Grow the colonies at 30 °C for 2–3 days.
2. For each transformation, select 3 single colonies and inoculate cultures in 500 μL liquid SC-U in 96-well culture square plates. Keep a careful map of the plates. Seal the plates with a gas-permeable seal. Incubate overnight with shaking at 30 °C.
3. Prepare large YPD agar plates containing increasing concentrations of salt as described above.
4. Resuspend the cells by pipetting and dilute 5 μL of the culture in 200 μL TE in 96-well plates as described above.
5. Drop 5 μL of the dilutions on the YPD agar plates as described above.
6. Let the droplets dry and incubate the plates at 30 °C. Check daily the growth of the clones.
7. About 60 % of the candidate yeast clones initially identified in the primary screen were confirmed at this step. The DNA was sequenced using MPK6_F and MPK6_R primers.

4 Notes

1. This number should be significantly higher on plates for yeast which had the opportunity to recombine a functional vector using the linearized plasmid and the PCR product (*see* Table 1). Yeast transformant number largely varies from one experiment to another. The ideal density of colonies on plates is obviously as much as possible with the limit to have separated colonies.

   The number of colonies to be replicated in order to saturate the screen may be easily calculated from the length of the mutated ORF and the expected mutation efficiency selected in the mutagenesis protocol.

2. In our experience, spontaneous reversion of yeast growth on salt medium often occurs but independently of mutations in MPK6. Therefore, it is important to daily follow the plates and retain candidate colonies only if they may be found at the coinciding position on several plates of the replication series. Depending on the NaCl concentration as well as the amount of yeast transferred on the plate medium, interesting colonies may appear after 3–4 days.

3. Make sure that the competency of the bacteria is high enough as often this step is limiting. Alternatively, commercial competent cells may be used.

4. To avoid preparing many plates and yeast dilutions, we usually resuspend the transformed yeast in 20 μL TE, spot them as a drop on the SC-U medium surface and use inoculator loops to spread the yeast in rough gradient of density to have separated colonies.

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