# Function of the C-terminal domain of Hkr1, a signaling mucin of *Saccharomyces cerevisiae* in the context of bud site selection and resistance to the cell wall integrity disruptor HM-1 killer toxin

Toshihiro Kondo, Ukyo Suzuki and Shin Kasahara*

Food Microbiology Unit, School of Food & Agricultural Sciences, Miyagi University, Sendai, JAPAN

*Correspondence; kasahara@myu.ac.jp

Lindnera mrakii (syn. *Williopsis saturnus* var. *mrakii* or *Hansenula mrakii*) produces a proteinous killer toxin called HM-1 which kills sensitive yeasts including *Saccharomyces cerevisiae*. *HKR1* (*Hansenula mrakii* killer toxin-resistant gene 1) was originally isolated from the genome of *S. cerevisiae* as a gene whose overexpression overcame the cytocidal effect of HM-1. The gene product Hkr1 is a large, highly glycosylated mucin-like type I transmembrane protein containing an N-terminal signal peptide sequence, Ser/Thr-rich repetitive sequences and a putative transmembrane domain. Calcium binding EF hand and leucine zipper motives were found in its cytoplasmic tail. It also functions as an osmosensor in the high osmolarity glycerol (HOG) MAP kinase pathway. We previously reported that only the partial sequence of Hkr1 endowed HM-1 resistance to the cells, then first in this study, the minimum sequence of Hkr1 required for HM-1 resistance was determined by serial deletions. Apparently the extracellular HMH (Hkr1-Msb2 Homology) domain in addition to the cytoplasmic tail was indispensable for HM-1 resistance. Also we observed that the cells overexpressing partial *HKR1* showed altered budding patterns. The haploid *S. cerevisiae* cells mainly select bud sites in an axial pattern, but bipolar and randomized patterns were often observed in the presence of HM-1. The mutant cells lacking the cytoplasmic part of Hkr1 showed an aberrant budding pattern, too. It is well studied that a series of *BUD* gene products, Bud1/Rsr1, Bud2, Bud5 and so forth are required for proper bud-site selection in *S. cerevisiae*. Since both overexpression and disruption of *HKR1* gave rise to abnormal budding patterns, Hkr1 might regulate budding coordinately with those proteins, or possibly interacting with some other factors. Moreover, we found that the budding pattern of haploid *S. cerevisiae* cells grown under the influence of HM-1 was also affected, suggesting that HM-1 perturbed the bud-site selection process. HM-1 has been studied as a cell wall integrity disruptor and believed to inhibit the synthesis of cell wall polysaccharides, but we now postulate that it may target other cellular events such as bud site selection and cell polarity regulation. Our observations could provide important pieces of information to understand the mechanism of the cytotoxicity of HM-1 and the function of Hkr1, especially its cytoplasmic domain in bud site selection as well as cell wall integrity of *S. cerevisiae*.
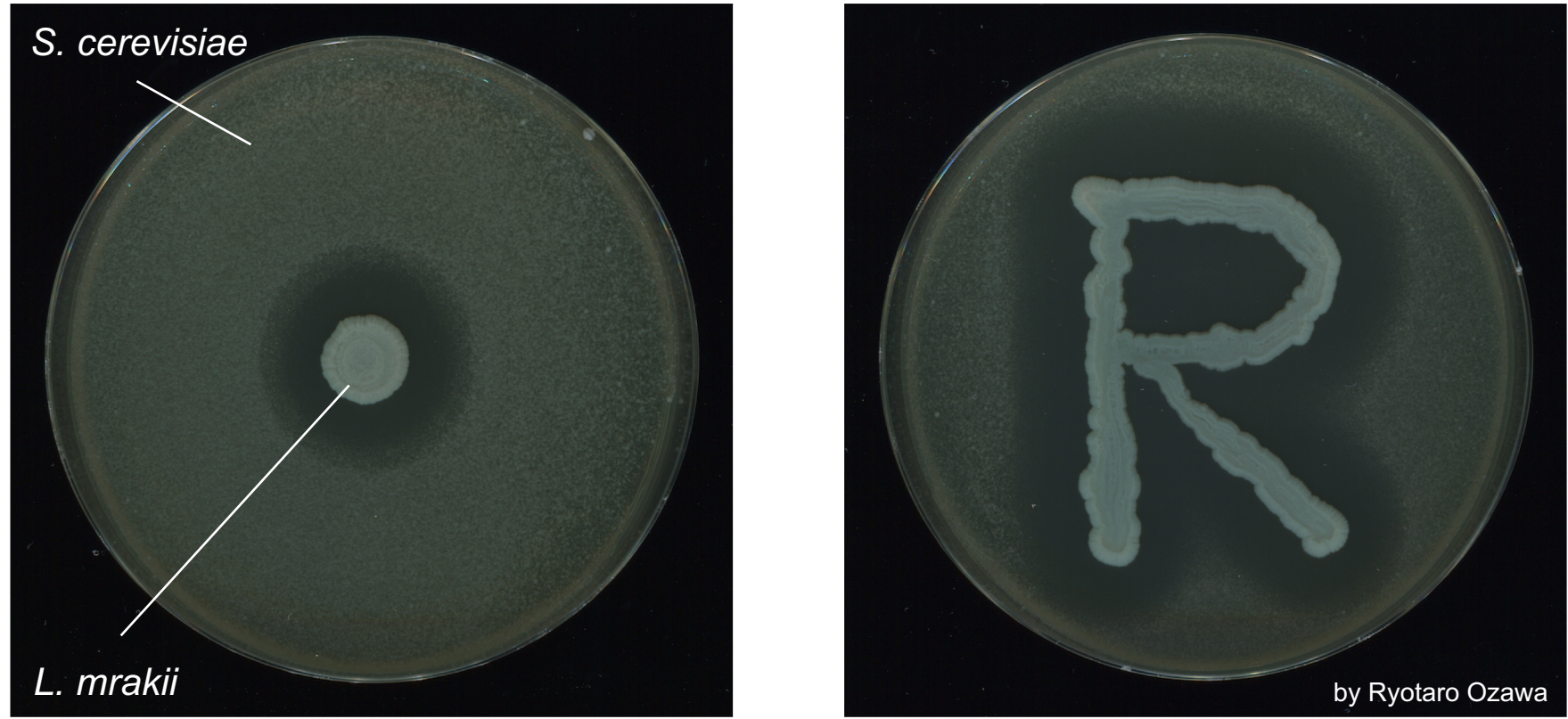

Fig. 1 The killer toxin called HM-1 produced by *L. mrakii* kills some other yeasts such as *S. cerevisiae*. See the clear zone around growing *L. mrakii*. HM-1 is known as a cell wall integrity disruptor for sensitive yeasts, also inhibits cell wall β-glucan synthesis. HM-1 is a relatively small protein, consists of 88 amino acids and is very stable at high temperature (over 100℃), high and low pH (4 to 11).
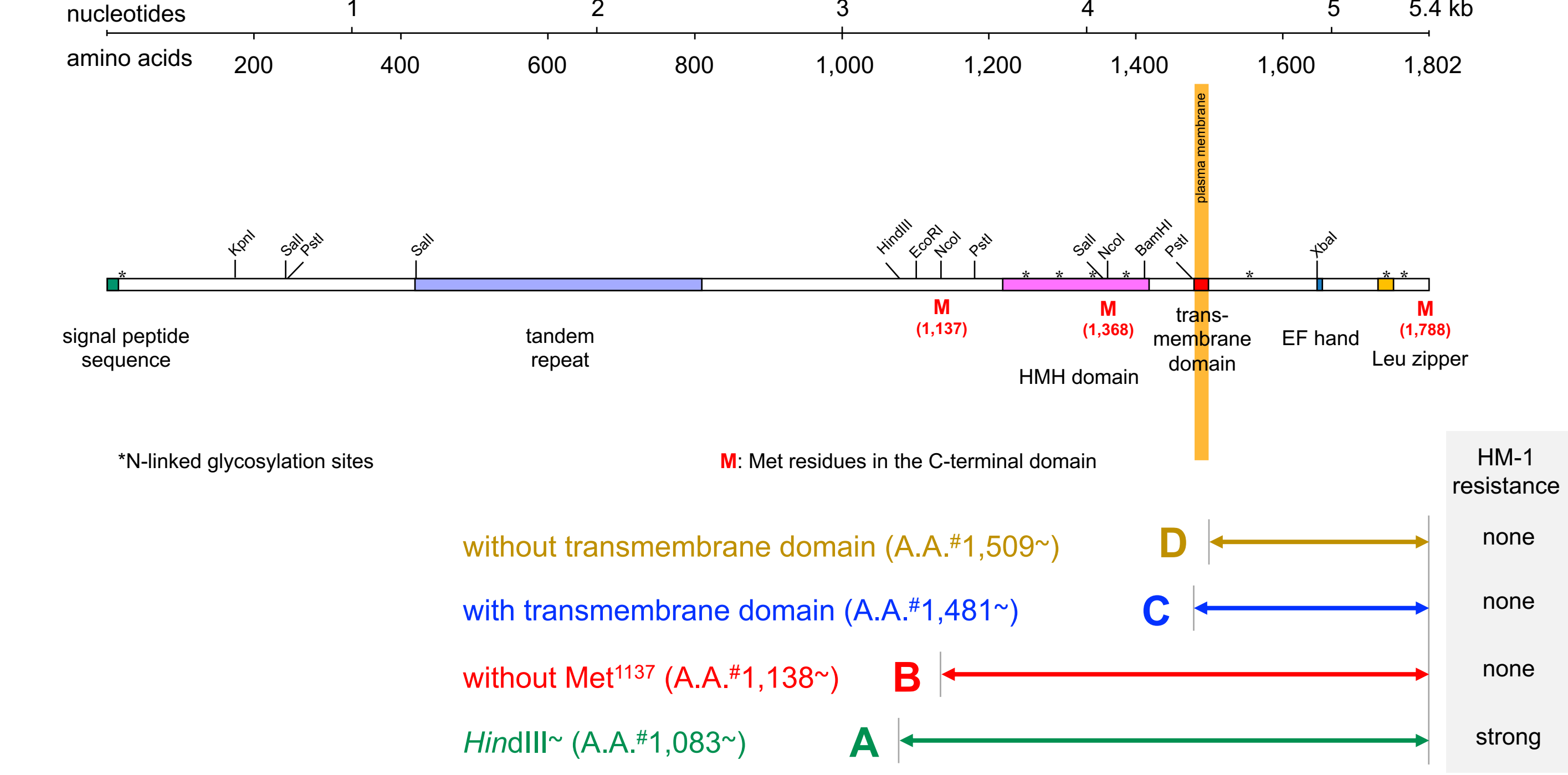

Fig. 2 Structure of the *S. cerevisiae* *HKR1* gene and the Hkr1 protein. Shown are the N-terminal signal peptide sequence, the extracellular tandem repeats which are commonly found in most mucins, the HMH domain, the highly hydrophobic transmembrane domain, the calcium-binding EF hand consensus and the leucine zipper motif. The asterisks indicate potential N-glycosylation sites and the internal methionine residues which could function as translational initiation sites in the C-terminal domain are marked as "M".

*HKR1*C-term., the C-terminal domain of *HKR1* (*Hind*III~ fragment of *HKR1* = **A**) and the shorten versions (**B**, **C** and **D**) are overexpressed in the haploid *S. cerevisiae* A451 cells by using the multicopy vector under the control of the *ADH1* promoter or the inducible *GAL1* promoter.

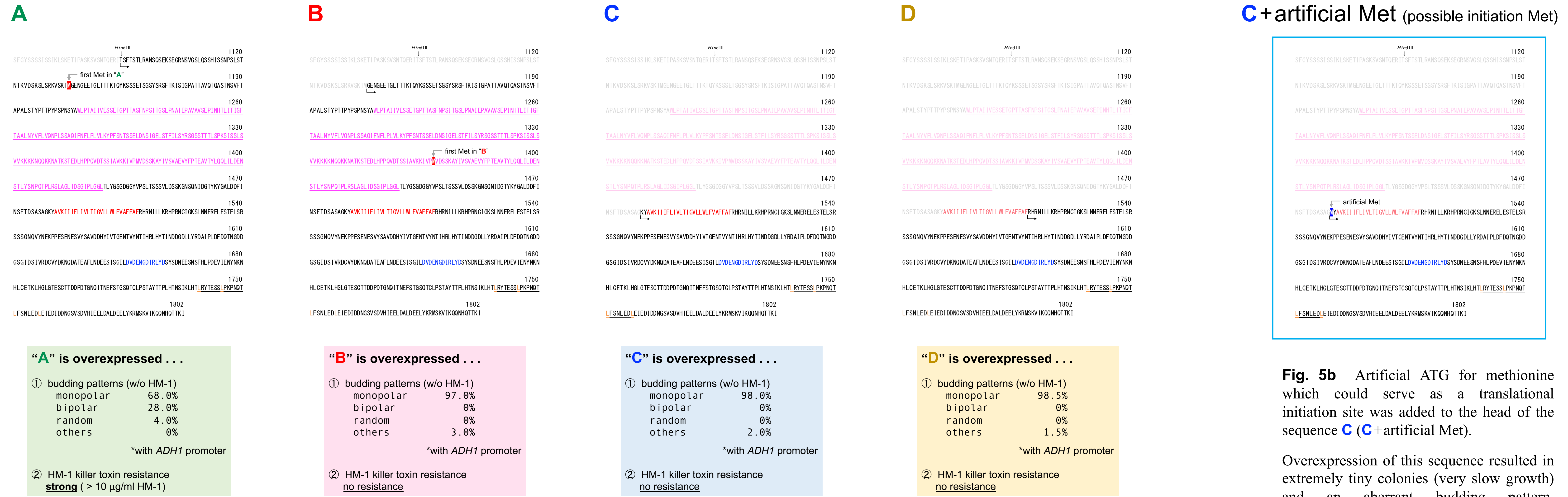Only the overexpression of the sequence **A** rescued the cells from the cytocidal effect of HM-1.
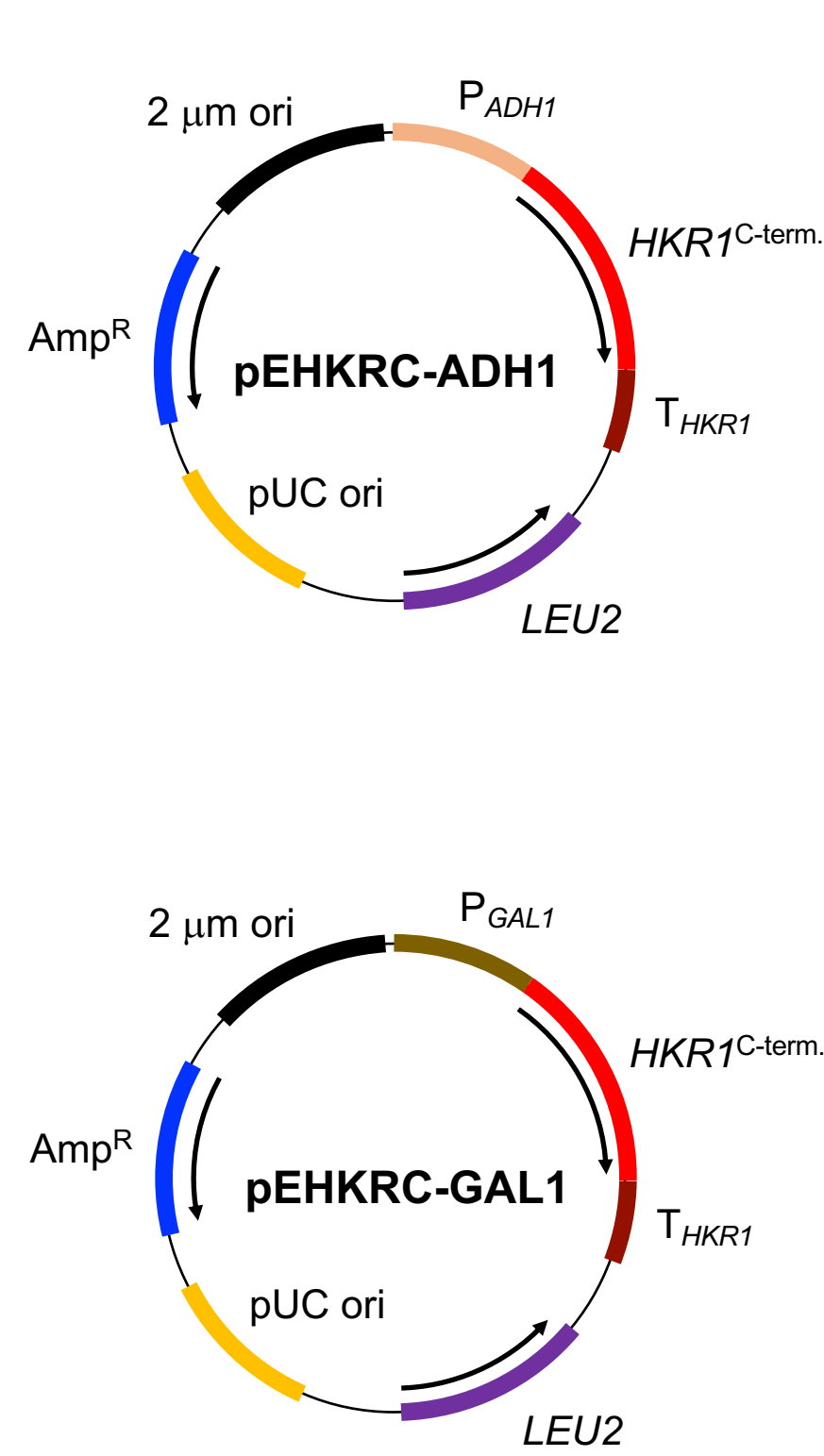
Fig. 3 Plasmid construction for the overexpression of *HKR1*C-term. (**A**) and the deleted derivatives (**B**, **C** and **D**, see Fig. 2). The constitutive *ADH1* promoter was utilized to transcribe the all inserts (pEHKRC-ADH1). The sequence **A** was overexpressed by using the inducible *GAL1* promoter (pEHKRC-GAL1). Both plasmid vectors carry the multicopy 2 μm replication origin and the *LUE2* gene as a selectable marker. For the expressions of **C** + artificial Met (see Fig. 5), only the vector pEHKRC-ADH1 was used.
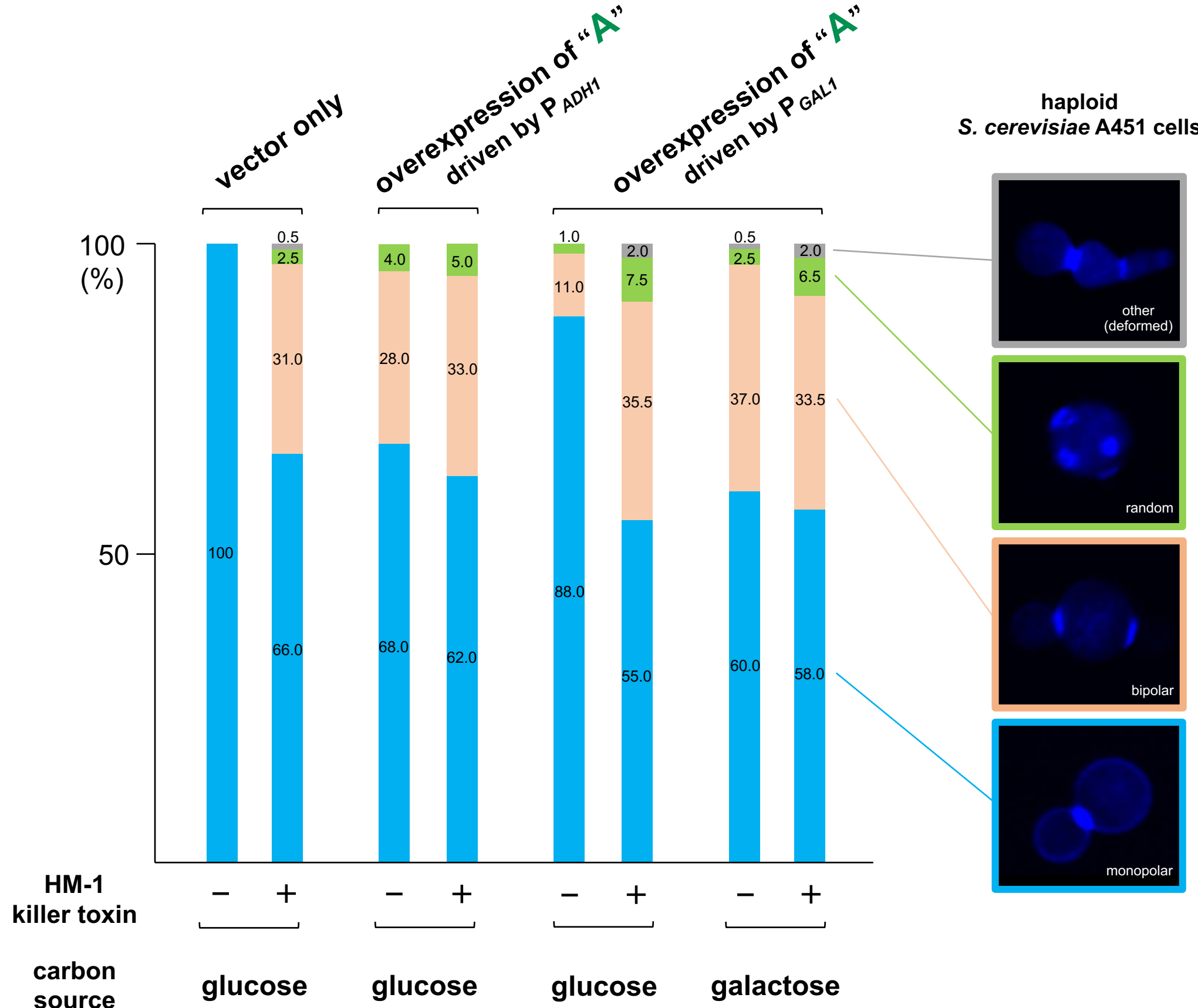

Fig. 4 HM-1 affects the budding patterns of *S. cerevisiae*. Haploid *S. cerevisiae* A451 transformed with the vector only, the vector+**A**, vector+**B**, vector+**C** and vector+**D** were incubated for 16 hours in the yeast nitrogen base (YNB) medium containing 2 μg/ml of HM-1 (equivalent of IC₅₀), stained with Calcofluor white, then observed under the fluorescence microscope. Cells were categorized into four budding/morphological patterns; "monopolar", "bipolar", "random" and "deformed". Two hundred cells were observed for each transformational/cultural condition. Transformants carrying the pEHKRC-GAL1 plasmid for *HKR1*C-term. overexpression with the *GAL1* promoter were cultured in the galactose-containing YNB medium. Otherwise the cells were incubated in the glucose-containing YNB medium. It is known that culturing haploid *S. cerevisiae* cells under glucose-limiting conditions (culturing with galactose) induces distal-unipolar (bipolar) budding, which is rarely observed in haploid strains.

S. cerevisiae A451 cells treated with HM-1 showed aberrant budding patterns. The haploid *S. cerevisiae* buds usually in a monopolar manner, but 34% of HM-1 treated cells propagated in different budding manners (31% bipolar, 2.5% random).

Cells overexpressing functional *HKR1* (**A**) also showed irregular budding patterns. Overexpression of **A** endowed strong resistance to HM-1, and such HM-1 resistant transformants cultured in the HM-1-containing medium did not recover a normal budding pattern. Overexpression of **B**, **C** and **D**, none of which rescued cells from the toxicity of HM-1, did not affected the budding pattern of *S. cerevisiae* A451.

"A" is overexpressed . . .

① budding patterns (w/o HM-1)
- monopolar 68.0%
- bipolar 28.0%
- random 4.0%
- others 0%

*with *ADH1* promoter

② HM-1 killer toxin resistance
strong (> 10 μg/ml HM-1)

"B" is overexpressed . . .

① budding patterns (w/o HM-1)
- monopolar 97.0%
- bipolar 0%
- random 0%
- others 3.0%

*with *ADH1* promoter

② HM-1 killer toxin resistance
no resistance

"C" is overexpressed . . .

① budding patterns (w/o HM-1)
- monopolar 98.0%
- bipolar 0%
- random 0%
- others 2.0%

*with *ADH1* promoter

② HM-1 killer toxin resistance
no resistance

"D" is overexpressed . . .

① budding patterns (w/o HM-1)
- monopolar 98.5%
- bipolar 0%
- random 0%
- others 1.5%

*with *ADH1* promoter

② HM-1 killer toxin resistance
no resistance

Fig. 5b Artificial ATG for methionine which could serve as a translational initiation site was added to the head of the sequence **C** (**C** + artificial Met).

Overexpression of this sequence resulted in extremely tiny colonies (very slow growth) and an aberrant budding pattern. Presumably the overexpression of the Hkr1 cytoplasmic tail was toxic and perturbed healthy budding.

Fig. 5a Each part of *HKR1* (**A**, **B**, **C** or **D**) was subcloned into the plasmid vector pEHKRC-ADH1 and introduced to *S. cerevisiae* A451. The insert **A** contains the C-terminus (*Hind*III~) of *HKR1* and the ATG (for Met1137) could be utilized as a translational initiation codon. The insert **B** contains the region corresponding to Gly1138 to the C-terminus of Hkr1 and is unable to use the ATG for Met1137, but another ATG for Met1368 could be used for translational initiation. The clones **C** and **D** lack the HMH domain. The clone **C** contains the sequence for the transmemrane domain, while the clone **D** does not.
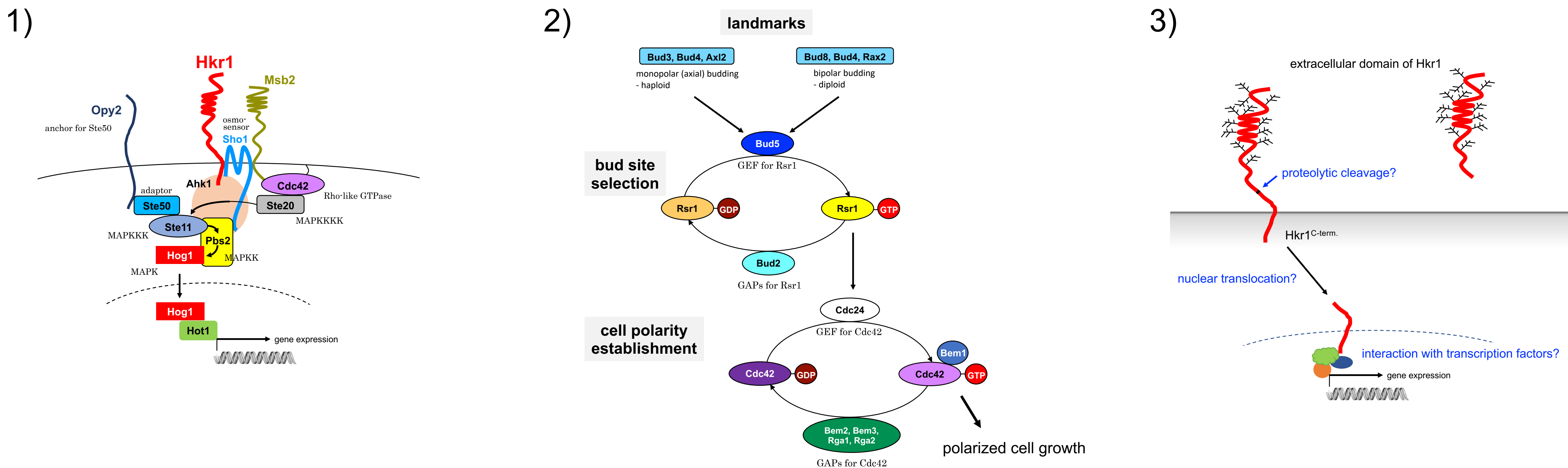
Fig. 6 1) Hkr1 is a membrane-bound signaling mucin that acts as an osmosensor in the HOG MAP kinase signaling. Major components and interactions among them are depicted.

2) Signaling components involved in polarization in budding in *S. cerevisiae*. Since both overexpression and disruption* of *HKR1* affected the budding pattern, involvement of Hkr1 in this complex, either direct or indirect, may be considered.

*Yabe, T. *et al.* (1996) *J. Bacteriol.* **178**(2), 477-483.

3) Similar to other mucinous proteins, it is likely that Hkr1 is first translated as a single polypeptide, then undergoes processing; possibly it is cleaved proteolytically at a certain position in its C-terminal domain, to release the tail to the cytosol.

While Hkr1 has been well studied as a component of HOG MAP kinase signaling, it is not clear for now that Hkr1 functions as a member of bud site selection machinery. Are those two different signaling pathways associated? Does Hkr1 link them? Does HM-1 perturb the signaling? We are currently working on these questions.