通过CRISPR/Cas9基因编辑手段构建白念珠菌 *CaHSL1*基因的失活突变体及其表型分析

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摘要 钙离子稳态和钙离子信号途径对白念珠菌的形态发生、耐药性以及毒力都十分重要。白念珠菌和酿酒酵母菌的细胞质膜蛋白质Rch1p均富集在母细胞和子细胞之间的芽颈部位, 负向调控胞外钙离子的内流。与细胞周期调控相关的蛋白激酶Hsl1p也存在于白念珠菌和酿酒酵母菌的芽颈部位。该研究发现, 抑制*CaHSL1*基因的表达或者通过CRISPR(clustered regularly interspaced short palindromic repeat)/Cas9新方法失活*CaHSL1*基因, 均可导致白念珠菌对钙离子敏感, 这种钙离子敏感性可以被钙调磷酸酯酶的专一性抑制剂环孢霉素抑制。这个结果表明, 钙离子稳态和钙信号转导途径可能与细胞周期调控有关。此外, *CaHSL1*基因失活突变体对十二烷基磺酸钠、氟康唑、酮康唑、刚果红、潮霉素B、卡泊芬净和Anidualafungin敏感, 表明CaHsl1p与细胞质膜和细胞壁胁迫的应答相关。

关键词 白念珠菌; *CaHSL1*; CRISPR; 钙离子; 细胞质膜压力; 细胞壁压力

Construction of Inactivation Mutant for *Candida albicans CaHSL1* with the Novel CRISPR/Cas9 Approach and Its Phenotype Analysis

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Abstract Calcium-homeostasis and calcium signaling pathway are important for the morphogenesis, drug tolerance and virulence of *Candida albicans*. In both *C. albicans* and *Saccharomycese cerevisiae*, the plasma membrane protein Rch1p is enriched at the bud neck between the mother cell and the daughter cell, while Hsl1p related to the control of cell cycle is also localized to the bud neck. In this study, the results show that conditional repression of *CaHSL1* expression or inactivation of *CaHSL1* expression through the novel approach CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 leads to calcium sensitivity of cells. This calcium sensitivity was suppressed by the block of calcium signaling through cyclosporin A, the specific inhibitor of calcineurin. This work suggests that calcium homeostasis and calcium signaling might be related to cell cycle control. In addition, the CRISPR/Cas9 mutant for *CaHSL1* is sensitive to sodium dodecyl sulfate, fluconazole, ketoconazole, congo red, hygromycin B, caspofungin and anidulafungin, indicating that CaHsl1p is involved in the

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response of *C. albicans* cells to cell membrane and cell wall stresses.

Keywords *Candida albicans*; *CaHSL1*; CRISPR; phenotypic analysis; cell plasma membrane and cell wall stress

白念珠菌是人体共生菌, 存在于口腔、生殖道以及胃肠道的黏膜[1]。随着近年来HIV阳性患者、接受理化治疗的癌症患者、器官移植患者等免疫功能低下人群的日益增加, 白念珠菌系统性感染这些患者导致的条件致病性越来越普遍[2-3]。大量研究表明, 细胞内钙离子稳态对白念珠菌应答外界环境压力、耐药性、形态发生和致病性有着至关重要的作用[4-6]。真核生物中, 钙离子/钙调磷酸酯酶信号途径是高度保守的[7]。白念珠菌细胞质膜上控制钙离子吸收的高亲和性钙离子通道(由Mid1p、Cch1p和Ecm7p组成)和低亲和性钙离子通道(Fig1p), 以及内质网和高尔基体膜上的钙泵(Spf1p和Pmr1p)和液泡膜上的钙泵(Pmc1p)已经被鉴定[8-13]。当细胞质内钙离子浓度低于正常范围时, 液泡膜上的钙离子通道蛋白质Yvc1p可以将液泡中的钙离子释放到细胞质中, 以维持细胞存活所需的钙离子浓度[14]。细胞质膜上的Rch1p是负调控钙离子内流的因子, 它的缺失导致胞内钙离子浓度增加和钙离子/钙调磷酸酯酶信号途径的激活, 造成细胞对钙胁迫高度敏感[15-16]。当细胞内钙离子浓度的升高时, 钙离子/钙调磷酸酯酶信号途径被激活调控钙离子泵在内的许多基因的表达来实现胞内钙离子稳态[17]。尽管我们目前已经对白念珠菌的钙离子信号途径及其多种组分有了基本认识, 但是白念珠菌的钙离子稳态调控机理方面的许多细节仍有待阐明[6]。

我们前期的工作发现, 细胞质膜蛋白质CaRch1p富集在白念珠菌母细胞和子细胞的芽颈部位[15]。此外, 酿酒酵母细胞中的同功蛋白ScRch1p在母细胞和子细胞完全分裂之前也会暂时富集在芽颈部位[18]。令人感兴趣的是, 酿酒酵母和白念珠菌细胞中四种蛋白激酶Hsl1p、Gin4p、Swe1p和Cdc28p都存在于母细胞和子细胞之间的芽颈部位, 参与细胞周期的调控[19]。白念珠菌GRACE(gene replacement and conditional expression)文库由2 358个基因的GRACE菌株组成, 这些基因的一个等位基因被*CaHIS3*替代, 另一个等位基因的表达则被四环素(tetracycline, Tet)启动子控制。在四环素存在条件下, 该等位基因的表达受到抑制, 因此, 目标基因的菌株表现出纯合子突变体的表型[20]。但是, 这个GRACE文库中没有*CaGIN4*和*CaSWE1*两个基因的菌株。由于*CaCDC28*是一个必需基因[21], 在四环素存在的条件下, *CaCDC28*基因的GRACE菌株就已经存在严重缺陷, 因此我们没有对它的GRACE菌株做进一步的研究。但我们对*CaHSL1*基因的GRACE菌株进行了钙离子敏感性的表型分析。和野生型菌株相比, *CaHSL1*的GRACE菌株在四环素存在的条件下对钙离子敏感(图1A)。

CRISPR(clustered regularly interspaced short palindromic repeat)/Cas9是一种新型基因编辑手段, 最近才应用于白念珠菌的基因研究[22-24]。为了进一步深入研究*CaHSL1*基因与钙离子敏感性的关系, 我们采用这种新型基因编辑手段对*CaHSL1*基因进行失活, 成功获得了*CaHSL1*的失活突变纯合子, 并对它进行了系统的表型分析。

1 材料与方法

1.1 菌种、质粒和引物

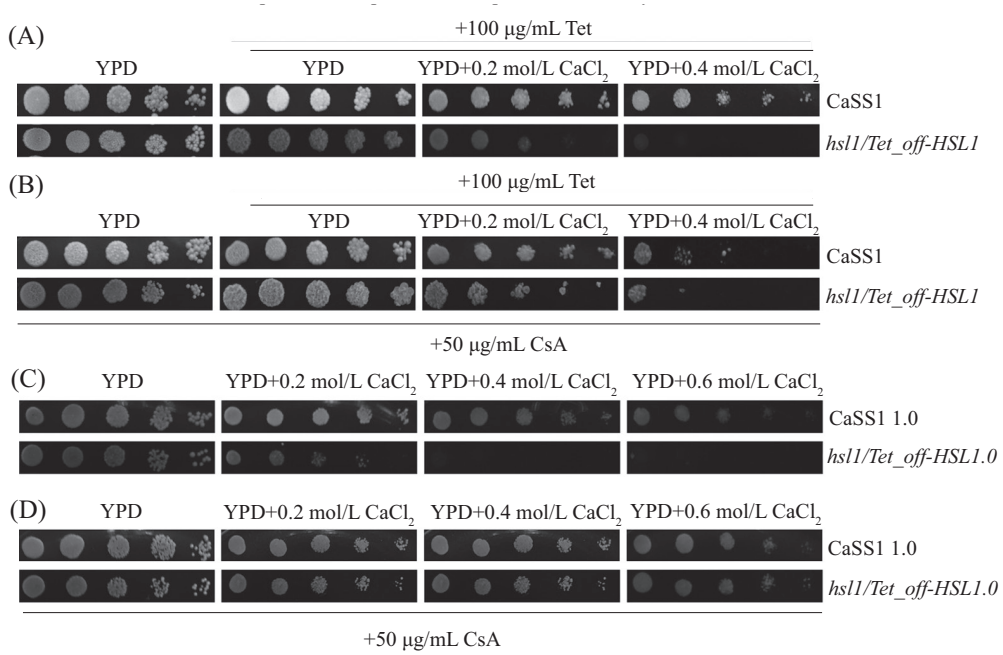
本实验所用菌株和质粒见表1, 引物见表2。

1.2 培养基

培养基 YPD培养基(液体和固体): 蛋白胨20 g、酵母提取物10 g、葡萄糖20 g, 去离子水定容至1 L(固体培养基另外需加入20 g琼脂), 121 ℃高压灭菌后4 ℃保存备用; LB培养基+氨苄青霉素(Ampicillin, Amp)(液体和固体): 蛋白胨10 g、酵母提取物5 g、氯化钠10 g、去离子水定容至1 L(固体培养基另外需加入20 g琼脂), 121 ℃高压灭菌后加入100 μg/mL的Amp, 4 ℃保存备用; SD-URA培养基(液体和固体): 酵母氮源(含硫酸铵)6.7 g、葡萄糖20 g、氨基酸混合物(苏氨酸200 mg、L-缬氨酸150 mg、L-色氨酸40 mg、L-蛋氨酸20 mg、L-酪氨酸30 mg、L-赖氨酸30 mg、L-异亮氨酸30 mg、L-苯丙氨酸50 mg、硫酸腺嘌呤20 mg、L-精氨酸20 mg、组氨酸20 mg、亮氨酸100 mg), 去离子水定容至1 L(固体培养基另外需加入20 g琼脂), 121 ℃高压灭菌后4 ℃保存备用。

1.3 试剂

PCR相关试剂、T4连接酶等试剂全部购自NEB



A、B: 分别为GRACE野生型CaSS1菌株和*hsl1/Tet_off-HSL1*菌株在不加和补加50 μg/mL CsA条件下的钙离子敏感表型。GRACE文库中*hsl1/Tet_off-CaHSL1*菌株*CaHSL1*基因的表达在四环素存在条件下被抑制。C、D: 分别为GRACE 1.0野生型CaSS1 1.0菌株和*hsl1/Tet_off-HSL11.0*菌株在不加和补加50 μg/mL CsA条件下的钙离子敏感表型。GRACE 1.0文库中*hsl1/Tet_off-CaHSL1*菌株*CaHSL1*基因即使在缺乏四环素的条件下也不能够表达。实验菌株先接种在液体YPD培养基里, 30 ℃摇床培养过夜。第二天对过夜培养物进行10倍的系列稀释。从原液及其四个稀释液中取2 μL点到试验平板上。平板在30 ℃培养箱中培养2~3天后拍照记录表型。

A,B: represent the calcium-sensitive phenotypes of the wild type CaSS1 and its isogenic mutant *hsl1/Tet_off-HSL1* in the absence or presence of 50 μg/mL CsA, respectively. *CaHSL1* expression in *hsl1/Tet_off-CaHSL1* strain from GRACE library is repressed in the presence of tetracycline. C,D: represent the calcium-sensitive phenotypes of the wild type CaSS1 1.0 and its isogenic mutant *hsl1/Tet_off-HSL1 1.0* in the absence or presence of 50 μg/mL CsA, respectively. *CaHSL1* expression in *hsl1/Tet_off-CaHSL1* strain from GRACE 1.0 library is always repressed even in the absence of tetracycline. Test strains were inoculated in liquid YPD and cultured overnight at 30 ℃. Overnight cultures were serially diluted by 10 times, and cells of 2 μL from each dilution were spotted onto plates. Plates were incubated at 30 ℃ for 2-3 days before photos were taken.

图1 GRACE和GRACE 1.0的*hsl1/Tet_off-HSL1*菌株对钙离子表型

Fig.1 Calcium-sensitive phenotypes of GRACE and GRACE 1.0 *hsl1/Tet_off -HSL1* strains

表1 菌株和质粒

Table 1 Strains and plasmids

名称 Name	基因型或描述 Phenotype of genomic or description	来源 Source
Strain		
CaSS1	CAI4 *HSL/HSL1 LEU2/leu2::hisG-URA3-hisG-tet*R-GAL4AD	[20]
Tet_off-HSL1	CAI4 *hsl1::HIS3/Tet_off-HSL1 LEU2/leu2::hisG-URA3-hisG-tet*R-GAL4AD	[20]
CASS1 1.0	CAI4 *HSL/HSL1 LEU2/leu2::hisG*	[25]
Tet_off-HSL1 1.0	CAI4 *hsl1::HIS3/Tet_off-HSL1 LEU2/leu2::hisG*	This study
SN148	*Arg4/arg4 leu2/leu2 his1/his1 ura3::imm434/ura3::imm434*	[26]
HHCA1	SN148 *hsl1/hsl1 Nat*^{R}	This study
HHCA2	SN148+pCR4	This study
HHCA3	SN148 *hsl1/hsl1 Nat*^{R}+ pCR4	This study
HHCA4	SN148 *hsl1/hsl1 Nat*^{R}+ pCR4-CaHSL1	This study
Plasmid		
pV1093	*Amp*^{R} *Nat*^{R}	[24]
pV1093-sgHSL1	*Amp*^{R} *Nat*^{R}	This study
pCR4	*Amp*^{R} *URA*	[16]
pCR4-CaHSL1	*Amp*^{R} *URA*	This study

表2 实验所用引物

Table 2 Primers used in this study

引物名称 Name of primer	引物序列 Sequence of primer
HSL1-sgF	ATT TGT GAC TCT CCG AGT AAT CAT TG
HSL1-sgR	AAA ACA ATG ATT ACT CGG AGA GTC AC
HSL1-RF	CAA TGT CAA CAG TTG TTA ATA GAC GGT CAT CAC ATC AGT TTT AAC TCT CCG AGT AAT CAT CTG CAG
HSL1-RR	GAT TGA ACC ACC TTA TCC ACA TTC ATC GAA GAT GAA TGA TCT GCA GAT GAT TAC TCG GAG AGT TAA
HSL1-CF	CCA ACC TTC ACT CAT CAA CG
HSL1-CR	CTG GTG AAG CAT AAT GAG GAG
LR159F	CCA AGA AGC ATC TAA TCA ACT CCC
LR159R	CGC GTC TAA GTT AGT CTC CGT TC
HSL1-clonF	GCC AAG CTT GCA TGC CTG ACG CAC ACA GAA GGC AAT TGG TTA CTA C
HSL1-clonR	TAC GAA TTC GAG CTC GGT ACC CAG GAT CAA GTG GAT GAA TTG TTG

下划线标注为*Pst* I酶切位点。

Under line marked sequence are *Pst* I restriction enzyme site.

公司; 诺尔丝菌素(nourseothricin, NAT)购自Werner BioAgents公司; 酵母转化用醋酸锂、鲑鱼精DNA (salmon sperm DNA, ssDNA)和聚乙二醇PEG 3350以及细胞裂解酶(lyticase)、氨苄青霉素(Ampicillin, Amp)、荧光增白剂(calcofluor white, CFW)、刚果红(Congo Red)、酮康唑(ketoconazole, KCZ)、潮霉素B(hygromycin B)、氟康唑(fluconazole, Flu)、卡泊芬净(caspofungin, Caspo)和anidualafungin(Anidua)购自Sigma公司; 氯化钙、氯化理和十二烷基磺酸钠(sodium dodecyl sulfate, SDS)购自国药集团; 引物由南京金斯瑞生物科技有限公司合成; ClonExpress® II重组酶购自南京诺唯赞生物科技有限公司。

1.4 实验仪器与装置

实验仪器包括: PCR仪(德国艾本德公司)、全温摇瓶柜(太仓强乐实验设备厂)、台式冷冻离心机(日本日立公司)、立式压力蒸汽灭菌锅(上海博迅实业有限公司医疗设备厂)、核酸电泳仪(北京六一仪器厂)、凝胶成像系统和酶标仪(美国Bio-Rad公司)。

1.5 CRISPR/Cas9方法失活白念珠菌基因*CaHSL1*

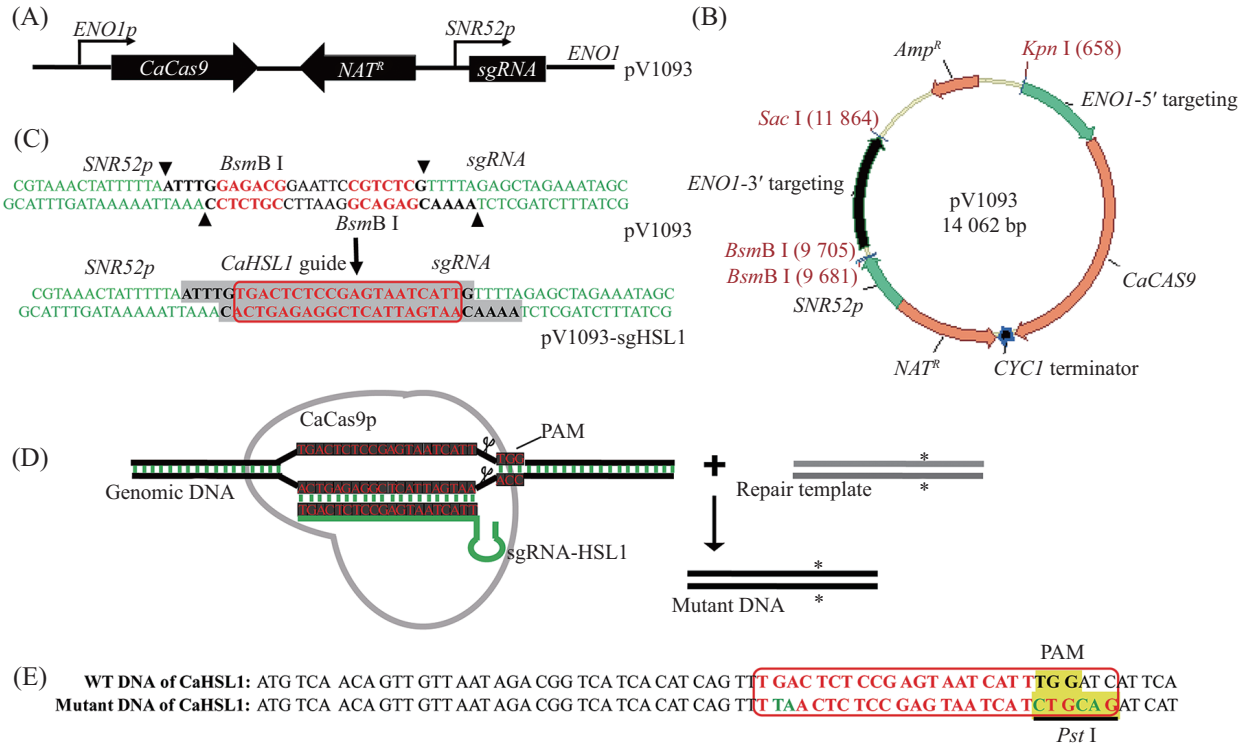
1.5.1 CRISPR/Cas9*方法原理* CRISPR体系是最初发现于细菌的一种免疫体系[26]。外源入侵的核酸序列初次入侵后可以被剪切并整合到细菌基因组特定位置上; 当同种病毒再次入侵时, 整合到基因组上的核酸序列表达, 与病毒DNA结合, 导致病毒被细菌的免疫系统识别, 细菌的Cas9蛋白快速识别对病毒基因组进行剪切。近年来, 这种体系已被改造运用于真菌、植物和动物的研究领域中[22-23]。白念珠菌是双倍体细胞, 自然界不存在单倍体形态, 敲除一个基因的两个等位基因需要分两步完成, 因此耗时和繁琐, 一定程度上制约了白念珠菌基因功能研究的速度。基于CRISPR技术的原理, Fink教授实验室[24]最近成功构建了一种在白念珠菌中可以操作的CRISPR体系, 可以对白念珠菌的基因组进行编辑, 使基因突变失活。细菌的Cas9p在白念珠菌细胞中不能正常表达, 通过密码子优化, 将细菌编码*Cas9p*基因的CTG密码子替换为CUG, 获得在白念珠菌细胞中正常表达且有功能的Cas9p蛋白, 并用*ENO1*启动子控制其表达(图2A和图2B)。Cas9p需要一个导向RNA(synthetic guide RNA, sgRNA)使其能够在基因组特定位点进行剪切。sgRNA是一段20 bp的核酸序列, 能够与基因组编辑位点序列进行杂交, 该序列后面紧跟着一个由NGG组成的PAM(protospacer-Adjacent Motif)位点, Cas9p能够识别该位点并在该位点进行剪切(图2C和图2D)。sgRNA的表达由白念珠菌RNA聚合酶III的启动子*SNR52p*控制(图2A和图2B)。

1.5.2 *引物设计* (1)导向RNA(synthetic guide RNA, sgRNA)引物。sgRNA是在PAM(NGG)位点前面20 bp。利用在线软件Benchling进行查找。选择

靠近起始密码子ATG以及On-Target和Off-Target分数都较高的sgRNA。由于sgRNA需要通过*Bsm*B I酶切位点连接到质粒pV1093上[24], 因此需要在正向引物加上5′-ATTTGX$_{20}$G-3′(表2, HSL1-sgF), 反向引物加上5′-AAAACX$_{20}$C-3′(表2, HSL1-sgR), PAM位点在正向引物3′端(图2C)。(2)修复DNA(repair DNA)引物。修复DNA是基因突变的模板, 含有突变序列和同源序列。突变序列包括三点修饰: (a)在基因ORF中插入终止密码子(UAA、UAG或UGA); (b)破坏PAM位点; (c)引入一个酶切位点, 来筛选正确的转化子, 选择酶切效率高且常用的限制性酶切位点(*Eco*R I、*Bam*H I、*Hind* III、*Nde* I、*Xba* I、*Xho* I、*Pst* I等)。同源序列是在正向和反向引物的5′端分别添加40 bp基因组上sgRNA两侧的序列(表2, HSL1-RF/HSL1-RR)。(3)筛选引物。在sgRNA两侧设计引物, 扩增出1 Kb左右的条带。酶切位点将这个片段分成两个容易区分片段(表2, HSL1-CF/HSL1-CR)。

1.5.3 sgRNA克隆 用限制性核酸内切酶*Bsm*B I酶切载体pV1093, 然后用CIP酶进行处理, 并用片段纯化试剂盒进行回收; 同时用T4 Polynucleotide kinase对sgRNA的引物进行退火添加磷酸基团。最后将sgRNA寡聚核苷酸片段与载体用T4 Ligase进行连接, 转化大肠杆菌感受态。

1.5.4 白念珠菌转化 用*Kpn* I和*Sac* I酶切克隆获得的质粒pV1093-sgRNA; 同时, PCR获得修复DNA, 最后对酶切质粒和修复DNA进行纯化回收。用



A: 由一个质粒pV1093组成的单一表达体系, 整合到基因组上*ENO1*基因位置。B: pV1093质粒图谱: *CaCas9*基因的3′末端融合有3X SV40核定位信号和3X FLAG标签; Cas9p的启动子是组成型表达的*ENO1*启动子, 同时是Cas9p整合到基因组上的整合位点。利用RNA聚合酶III的启动子SNR52p来表达sgRNAs。C: 用*Bsm*B I酶切质粒pV1093, 同时将设计的guide序列(红色框架内的*HSL1* guide序列)退火, 并将退火后的寡核苷酸(阴影部分序列) 连接到质粒上构建Guide表达系统。D: Cas9突变方法的原理图; 这种系统能够在基因的(*: PAM位点)位点产生纯合子突变, 同时使序列突变来阻止在整合后重复剪切。E: 野生型和突变株在*HSL1*突变位点的序列; 两个连续的终止密码子在*HSL1* ORF的阅读框架上; 在PAM位点引入了*Pst* I限制性酶切位点。

A: the solo system consists of one plasmid, pV1093, which targets *ENO1*. B: profile of plasmid pV1093. The *Cas9* gene is fused to sequences encoding the 3X SV40 nuclear localization signal and 3X FLAG tag for in-frame fusion to the 3' end of the gene. The Cas9p from this construct is expressed from the constitutive *ENO1* promoter at the plasmid integration site. The RNA polymerase III (Pol III) promoter *SNR52p* was used to express sgRNAs. C: guide expression system permit rapid cloning by digestion with *Bsm* BI followed by ligation of annealed oligos (shaded sequences) with desired guide sequence (HSL1 guide sequence in red box). D: schematic of Cas9 mutagenesis method. The system can create homozygous mutations in the gene (*: PAM site) and simultaneously mutate sequences to prevent repeated cleavage subsequent to integration. E: DNA sequences of *HSL1* locus in wild-type and mutant isolates. Two consecutve stop codons are on frame within *HSL1* ORF. *Pst* I restriction enzyme site is introduced at PAM region.

图2 白念珠菌CRISPR/Cas9系统

Fig.2 *Candida albicans* CRISPR/Cas9 system

LiAC转化法转化白念菌株细胞, 涂布在200 μg/mL NAT的YPD平板上进行筛选, 30 ℃培养2天, 将获得的转化子在200 μg/mL NAT的YPD平板上划线纯化, 30 ℃培养2天后提取基因组, 通过PCR获得目的片段后, 进行酶切验证。

1.6 pCR4-CaHSL1回复质粒构建实验

将pCR4质粒用*Kpn* I和*Pst* I进行双酶切, 通过PCR扩增*CaHSL1*的启动子、ORF以及终止子, 所用引物为HSL1-clonF/HSL1-clonR, 用ClonExpress® II重组酶将片段重组连接到pCR4载体上[16]。

1.7 表型实验

挑取单菌落接种到3 mL YPD培养基中, 30 ℃ 220 r/min过夜培养16 h, 调整*D*值为2, 10倍等梯度稀释到细胞浓度为10^3数量级。吸取2 μL, 点到YPD和含有不同药物的固体培养基上, 在30 ℃培养箱中培养2~4天, 拍照观察结果。

1.8 GRACE 1.0菌株*hsl1/Tet_off-HSL1 1.0*的构建

为了构建不受四环素控制的GRACE 1.0菌株*hsl1/Tet_off-HSL1 1.0*, 把GRACE菌株*hsl1/Tet_off-HSL1*接种在液体YPD培养基中培养过夜, 将过夜培养物的浓度稀释到10^3~10^4, 然后取100 μL在含有1 mg/mL 5-FOA的YPD平板上进行涂布, 获得丢掉URA3标记的转化子。把转化子在YPD和SD-URA平板上划线验证其基因型, 在YPD上能正常生长但是在SD-URA上不能生长的转化子, 就是成功从基因组上挤掉*URA3*标记和*tet*R-GAL4的正确转化子。

2 结果

2.1 抑制*CaHSL1*基因的表达导致白念珠菌细胞对钙离子敏感

Whiteway实验室最近构建了含有887个非必需基因的GRACE 1.0文库, 这些基因不受四环素抑制启动子的控制, 它们的表达一直处在被抑制的状态, 这样避免了四环素可能和筛选化合物产生相互作用的潜在弊端, 有利于菌株表型的筛选[25]。但是, GRACE 1.0文库没有包括*HSL1*基因的菌株。为了验证图1A和图1B中*HSL1*基因的GRACE菌株钙离子敏感表型, 按照以前构建GRACE 1.0文库的方法[25], 获得了*HSL1*基因的GRACE 1.0菌株。与它的GRACE菌株一样, 它也表现出对钙离子的敏感(图1C)。*HSL1*基因的GRACE菌株和GRACE 1.0菌株的钙离子敏感表型都可以被钙调磷酸酯酶的专一性抑制剂环孢霉素(CsA)抑制。这个结果表明, *HSL1*基因缺失导致的钙离子敏感表型是由于白念珠菌细胞内钙信号转导途径的激活造成的。

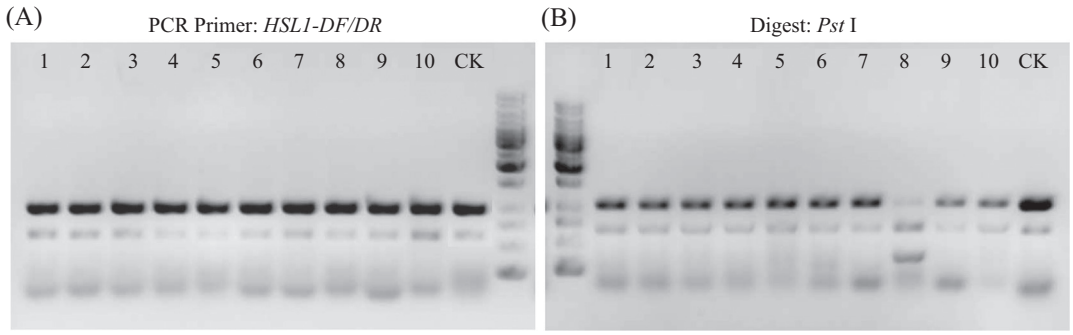
2.2 CRISPR方法获得的*CaHSL1*基因失活突变株的表型分析

为了进一步验证以上的钙离子表型, 我们通过一种新型的CRIPSR/Cas9手段构建*CaHSL1*基因的失活突变体。首先在Fink教授实验室[26]构建的白念珠菌CRISPR/Cas9体系载体pV1093中, 构建含有sgRNA的质粒pV1093-sgHSL1(图2C)。再将质粒pV1093-sgHSL1用*Kpn* I和*Sac* I双酶切线性化后, 与修复DNA片段同时转化白念珠菌野生型细胞SN148[26]。从获得的转化子基因组DNA中, PCR扩增出一条1 Kb的条带(图3A)。由于在设计修复DNA时, 引入了*Pst* I酶切位点, 所以产生突变的转化子在用*Pst* I进行单酶切后, 能够获得700和300 bp两个片段; 而野生型的基因组片段不含有该酶切位点, 酶切后仍然显示1 Kb的PCR片段。我们的8号转化子是带有预期突变的正确转化子(图3B), 并且通过进一步DNA测序得到验证。因此, 我们获得了*CaHSL1*的纯合子突变株。

与野生型SN148菌株相比, *CaHSL1*突变株虽然对低浓度的钙离子(0.2 mol/L)没有表型, 但是对0.4 mol/L和0.6 mol/L钙离子浓度敏感, 且这种钙离子敏感性可以被CsA抑制(图4)。这个结果与以上*CaHSL1*的GRACE菌株和GRACE 1.0菌株的表型结果基本一致(图1)。此外, *CaHSL1*突变株还对0.01% SDS、8 μg/mL Flu、8 μg/mL KCZ、500 μg/mL CR、100 μg/mL Hyg B、100 μg/mL Anidua、0.25 μg/mL Caspo等细胞壁和细胞膜胁迫药物敏感(图4)。在*CaHSL1*突变株中通过pCR4-CaHSL1导入*CaHSL1*基因, 可以回复它的表型(图4)。综上所述, HSL1p参与钙离子稳态以及细胞壁和细胞膜胁迫的应答。

3 讨论

Hsl1p是一个蛋白激酶, 通过调控Swe1p和Cdc28p来调控细胞周期[19,27]。本研究首次发现, CaHsl1p与白念珠菌细胞的钙离子敏感性相关, 缺乏*CaHSL1*基因的细胞内钙离子信号途径被持续性激活而导致其对钙离子敏感。我们实验室前期研究发现, 细胞质膜上的钙内流负调控因子Rch1富集在母

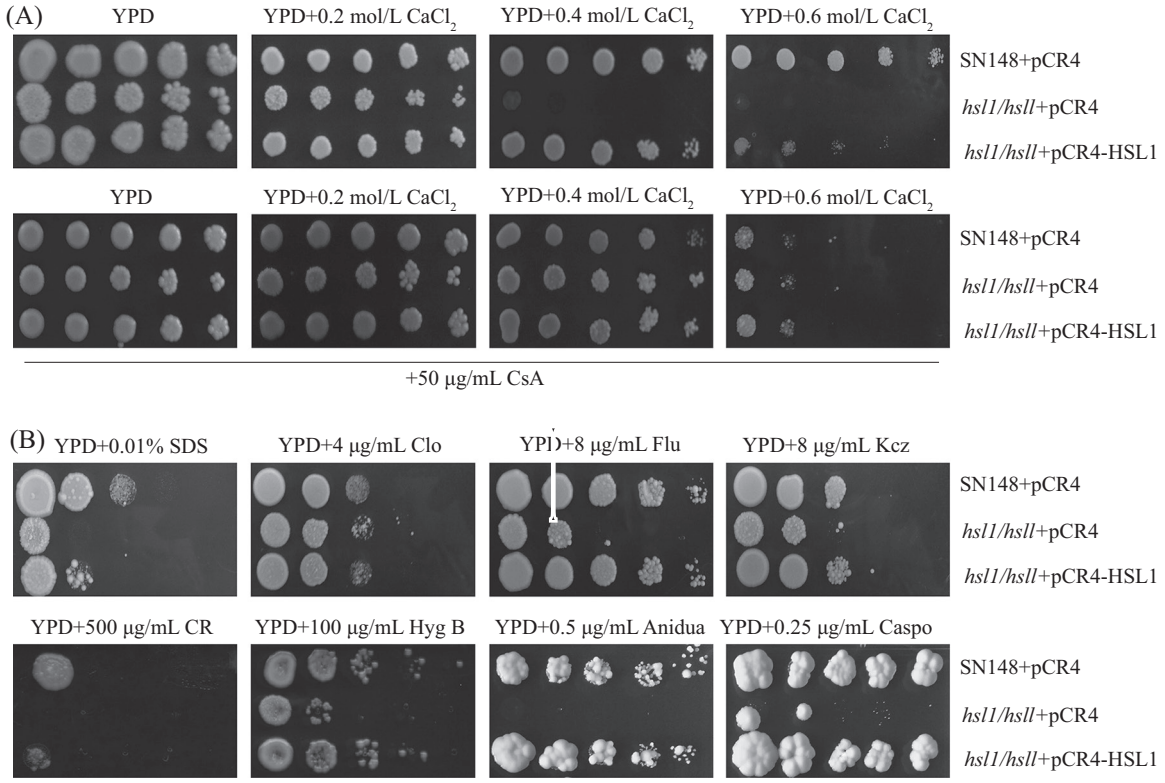


A: 转化获得转化子后, 菌落PCR扩增目的片段, 包含被突变位点。B: 修复DNA中引入酶切位点*Pst* I, 用限制性核酸内切酶*Pst* I对PCR产物进行酶切, 筛选正确的转化子。1~10代表10个不同的转化子。CK: 对照组。

A: colony PCR was used to amplify the flanking region including mutant site. B: to verify correct transformants, the PCR products were subjected to digestion with the restriction endonuclease *Pst* I whose site was introduced during the mutagenesis. No.1 to No.10 represent 10 different transformants. CK: control group.

图3 *CaHSL1*的纯合子失活突变株的基因型验证

Fig.3 Genotype confirmation of the homozygous inactivation mutant for *CaHSL1*



实验菌株先接种在液体SD-URA培养基里, 30 ºC摇床培养过夜。第2天对过夜培养物进行10倍的系列稀释。从原液及其四个稀释液中取2 μL点到试验平板上。平板在30 ºC培养箱中培养2~3天后拍照记录表型。

Test strains were inoculated in liquid SD-URA medium and cultured overnight at 30 ºC. Overnight cultures were serially diluted by 10 times, and cells of 2 μL from each dilution were spotted onto plates. Plates were incubated at 30 ºC for 2-3 days before photos were taken.

图4 *CaHSL1*的CRISPR突变株表型

Fig.4 Phenotypes of the CRISPR mutant for *CaHSL1*

细胞和子细胞之间的芽颈部位[15,18]。蛋白激酶Hsl1p也定位在芽颈部位[27-28]。因此, 母细胞和子细胞之间的芽颈部位的钙离子稳态可能与细胞的分裂相关。此外, 和酿酒酵母细胞*HSL1*基因缺失株的表型一样[29-31], 我们的结果表明, *CaHSL1*失活突变株对咖啡因、氟康唑、荧光白、刚果红等化合物敏感(图4)。刚果红是诱发细胞壁胁迫的药物[32], 它能够与多种多聚糖物质结合, 尤其对几丁质和β(1,3)-葡聚

糖具有高亲和性, 这两种染料都能够诱导不正常的隔膜, 从而导致母细胞和子细胞在细胞分裂过程中不能完全断裂。这些结果表明, 酵母Hsl1p参与细胞壁和细胞膜胁迫的应答反应。

CRISPR/Cas9是一种新的基因组编辑技术, 我们通过这一手段成功构建了白念珠菌*CaHSL1*失活突变株, 对*CaHSL1*基因的序列进行编辑, 导致*CaHSL1*基因的翻译提前终止而不能表达全长的CaHsl1蛋白质。*CaHSL1*失活突变株的钙离子表型和它的GRACE菌株一致(图1和图4)。但是, 我们发现, SN148背景菌株里构建的*CaHSL1*失活突变株对钙离子没有在CAI4背景菌株里构建的GRACE菌株那么敏感, 这可能是由于不同的菌株背景造成的。不同菌株背景下构建的*CaRCH1*基因缺失株对钙离子敏感性方面也存在差异[16]。我们的研究结果进一步确认了CRISPR/Cas9方法在白念珠菌基因功能研究领域的可行性和可靠性。

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