



MoLcb3 参与调控稻瘟病菌鞘脂平衡和胁迫反应

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摘要: 鞘氨醇 1-磷酸(sphingosine-1-phosphate, S1P)是一种具有生物活性的鞘脂, 因其参与各种生物过程的调节和许多疾病的发展而引人注目, 鞘氨醇 1-磷酸磷酸酶(S1P phosphatase, S1PP)在控制 S1P 胞内代谢起着重要作用, 而其在植物病原真菌中的生物学功能尚无报道。【目的】探究稻瘟病菌(*Magnaporthe oryzae*)鞘氨醇 1-磷酸磷酸酶在形态分化、致病过程和维持鞘脂平衡的作用。

【方法】利用同源重组方法敲除稻瘟病菌鞘氨醇 1-磷酸磷酸酶编码基因 *MoLCB3*, 获得 $\Delta MoLcb3$ 突变体, 并通过表型分析、基因互补、脂质代谢组学分析等对 *MoLcb3* 的生物学功能进行研究, 同时在 $\Delta MoLcb3$ 突变体中敲除稻瘟病菌鞘氨醇激酶(sphingosine kinase, SK) *MoLcb4*, 进一步探究磷酸酶 *MoLcb3* 和激酶 *MoLcb4* 之间的关系。【结果】敲除 *MoLCB3* 基因导致稻瘟病菌菌丝生长速率和产孢量显著下降, 影响分生孢子畸形率和附着胞初期形成, $\Delta MoLcb3$ 突变体完全丧失对大麦的致病性。 $\Delta MoLcb3$ 突变体在应对高渗透胁迫、细胞壁完整性胁迫、高温胁迫, 以及真菌脂质合成抑制剂三唑酮和多球壳菌素时, 与野生型有显著差异, 说明 *MoLcb3* 参与上述胁迫反应和脂质合成代谢。 $\Delta MoLcb3\Delta MoLcb4$ 双敲突变体可基本互补 $\Delta MoLcb3$ 突变体所有表型缺陷。另外, 脂质代谢组学分析显示, 与野生型相比, $\Delta MoLcb3$ 突变体部分脂质含量有显著差异, 例如游离脂肪酸、神经酰胺、磷脂酰肌醇等。【结论】鞘氨醇 1-磷酸磷酸酶 *MoLcb3* 在菌丝生长、产孢、孢子萌发、致病性、胁迫应激反应和维持脂质稳态等过程中起着重要作用, 此外敲除 *MoLCB4* 基因能缓解 *MoLcb3* 缺失带来的影响。本研究的结果为进一步阐明稻瘟病菌鞘脂代谢通路以及真菌脂质生物合成抑制剂的开发提供新的思路。

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MoLcb3 contributes to sphingolipid balance and stress responses in *Magnaporthe oryzae*

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Abstract: Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid notable for its involvement in the regulation of biological processes and the development of diseases. Sphingosine-1-phosphate phosphatase (S1PP) plays a role in regulating the intracellular metabolism of S1P, while the biological roles of S1PP in plant pathogenic fungi have not been reported. **[Objective]** To explore the role of S1PP in the morphological differentiation, pathogenic process, and maintenance of sphingolipid balance of *Magnaporthe oryzae*. **[Methods]** We employed homologous recombination to delete the S1PP gene *MoLCB3* from *M. oryzae* and characterized the obtained mutant $\Delta MoLcb3$ by phenotypic analysis, gene complementation, and lipid metabolomics. Furthermore, we deleted the sphingosine kinase (SK) gene *MoLcb4* from $\Delta MoLcb3$ to explore the relationship between MoLcb3 and MoLcb4. **[Results]** The deletion of *MoLCB3* resulted in significant decreases in the mycelial growth rate and spore production and affected conidial malformation and initial appressorium formation. $\Delta MoLcb3$ completely lost the pathogenicity to barley. Moreover, the $\Delta MoLcb3$ mutant were significantly different from the wild type in responding to hyperosmotic stress, cell wall integrity stress, high temperature stress, and fungal lipid synthesis inhibitors triadimefon and myriocin, suggesting that MoLcb3 was involved in these stress responses and lipid anabolism. Interestingly, the double mutant $\Delta MoLcb3\Delta MoLcb4$ basically compensated for all phenotypic defects of $\Delta MoLcb3$. In addition, lipid metabolomics showed that compared with the wild type, $\Delta MoLcb3$ presented significantly different levels of lipids, such as free fatty acids, ceramides, and phosphatidyl inositol. **[Conclusion]** MoLcb3 plays an important role in the mycelial growth, sporulation, spore germination, pathogenicity, stress responses, and lipid homeostasis. In addition, knockout of *MoLCB4* can cushion the effects of *MoLcb3* deletion. The results of this study provide new ideas for elucidating the sphingolipid metabolic pathway of *M. oryzae* and the development of inhibitors of fungal lipid biosynthesis.

Keywords: *Magnaporthe oryzae*; MoLcb3; sphingolipid; functional analysis; lipid metabolism

稻瘟病菌在水稻种植过程中引起毁灭性的病害——稻瘟病(rice blast), 导致全球粮食严重减产^[1-2], 极大地影响了世界粮食安全^[3-4]。多年来, 稻瘟病菌由于基因组测序完成早, 对功能遗传学的适应性以及在实验室环境中易验证结果而成为研究植物与病原菌相互作用的模式真菌^[5]。稻瘟病菌和水稻相互作用是一个高度动态和受调控的过程, 探究稻瘟病菌中能够影响致病性或毒力的途径, 以及如何克服水稻寄主免疫一直是研究的热点^[6-7]。近年来, 高渗透压甘油丝裂原活化蛋白激酶(high-osmolarity glycerol mitogen-activated protein kinase, HOG-MAPK)信号通路^[8]、细胞壁完整性(cell wall integrity, CWI)通路^[9]、细胞自噬途径^[10]和稻瘟病菌效应子调控机制^[11-12]等都得到了广泛的研究。尽管目前这几条途径对稻瘟病菌致病作用的调控机制已经基本清晰, 但是否还有其他途径参与调控稻瘟病菌的致病性仍然还不明确^[11]。

鞘脂是一类在自然界中广泛存在的脂质, 作为细胞膜的结构成分和信号分子具有重要的作用^[13-14]。鞘脂首先在内质网(endoplasmic reticulum, ER)中合成, 并被运输到高尔基体, 形成复杂鞘脂整合到质膜(plasma membrane, PM)中, 发挥免疫、抵抗非生物胁迫和通过胞间连丝的细胞间通讯等功能^[15-16]。真菌中富含鞘脂的质膜形成隔室, 有助于维持多种细胞功能和体内平衡^[17]。寄主鞘脂是寄主免疫反应的关键调节因子, 也有少量研究发现, 真菌鞘脂能促进真菌的致病性^[18-19]。

在动物、植物和真菌中, 鞘脂生物合成的主要过程具有一定的相似性, 其合成过程都是在真核生物细胞的内质网和高尔基体上进行的^[20]。神经酰胺(ceramide, Cer)是最简单的鞘脂类化合物^[21]。神经酰胺的生物合成主要分为4步。首先, 丝氨酸棕榈酰转移酶(serine palmitoyl transferase,

SPT)催化丝氨酸和棕榈酰辅酶 A, 缩合生成 3-酮基二氢鞘氨醇(3-ketodihydrosphingosine, 3-KDS)^[22]。然后, 3-KDS 经 3-酮基二氢鞘氨醇还原酶(3-ketodihydrosphingosine reductase, KDSR)还原生成二氢鞘氨醇(dihydrosphingosine, DHS)^[23]。接着, 二氢鞘氨醇与脂肪酸在神经酰胺合成酶(ceramide synthase, CerS)的催化下以酰胺键结合的形式, 形成二氢神经酰胺(dihydroceramide, dhCer)^[24]。随后二氢神经酰胺在二氢神经酰胺去饱和酶(dihydroceramide desaturase, DES)作用下合成神经酰胺^[25]。以上3个过程都是在内质网上完成的^[20]。最后, 神经酰胺通过囊泡或神经酰胺转运蛋白(ceramide transport protein, CERT)转运到高尔基体中与各种极性头部结合, 再转化为其他复杂鞘脂类物质^[26-28]。鞘氨醇(sphingosine, Sph)还能被鞘氨醇激酶磷酸化, 形成鞘氨醇-1-磷酸, 再通过鞘脂降解途径最终酶——鞘氨醇-1-磷酸裂解酶(sphingosine-1-phosphate lyase, SPL)分解, 产生乙醇胺磷酸盐(phosphoryl-ethanolamine)和 C16 脂肪醛(palmitaldehyde)^[29-30]。鞘氨醇-1-磷酸磷酸酶也能催化鞘氨醇-1-磷酸去磷酸化为鞘氨醇, 这是鞘氨醇激酶的逆反应^[31]。

鞘氨醇-1-磷酸磷酸酶最早是 de Ceuster 等在大鼠肝脏细胞膜中进行生化鉴定, 由 Mao 等和 Mandala 等从酿酒酵母(*Saccharomyces cerevisiae*)中克隆出来^[32-34]。S1PP 能分别将二氢鞘氨醇-1-磷酸、鞘氨醇-1-磷酸和植物鞘氨醇-1-磷酸(phytosphingosine-1-phosphate, PHSP)去磷酸化为二氢鞘氨醇、鞘氨醇和植物鞘氨醇(phytosphingosine, PHS)^[35]。在哺乳动物中, 两个同源基因 *SGPP1* 和 *SGPP2* 编码了特定的 S1P 磷酸酶, 与其他具有广泛底物特异性的脂质磷酸酶不同, S1P 磷酸酶对鞘氨醇磷酸盐具有高度底物特异性^[34,36]。当小鼠缺失 *SGPP1* 基因时, 角

质形成细胞中的 S1P 特异性的提高, 触发角质形成细胞过早分化, 导致出生后几天内患鱼鳞病并死亡^[37]。SGPP1 还参与了七氟烷抑制结肠癌细胞活力和促进细胞凋亡^[38], 而 SGPP2 能够调节小鼠胰岛细胞内质网的应激和增殖^[39]。同时, *LCB3* 编码的 S1PP 在酿酒酵母生长和热应激反应中起重要调控作用, 是将外源长链碱基并入鞘脂的关键基因^[33,40]。目前, *LCB3* 基因是否可以影响植物病原真菌的生长发育和致病性尚无报道。

本研究以 *MoLcb3* 为研究对象, 拟通过表型分析和脂质组学分析, 研究 *MoLcb3* 在调节稻瘟病菌生长发育、分生孢子萌发、致病性、胁迫应激和脂质稳态中的功能。此外通过在 $\Delta MoLcb3$ 突变体中敲除稻瘟病菌鞘氨醇激酶 *MoLcb4*, 明确 *MoLcb3* 和 *MoLcb4* 两者间的关系, 为进一步解析稻瘟病菌鞘脂代谢通路以及开发真菌脂质生物合成抑制剂的研究提供一些基础理论依据。

1 材料与方 法

1.1 材 料

稻瘟病菌野生型菌株 Guy11 及实验所用的感病水稻品种‘CO-39’种子、感病大麦‘Golden Promise’种子, 含敲除载体质粒 pKO3A 以及含互补载体 pKD5 的大肠杆菌(*Escherichia coli*), 由浙江省农业科学院植物保护与微生物研究所的真菌与植物免疫研究室保存, $\Delta Molcb3$ 突变体、 $\Delta Molcb4$ 突变体、互补菌株 $\Delta Molcb3-C$ 和 $\Delta Molcb3\Delta Molcb4$ 双敲突变体为本研究中构建。完全培养基(complete medium, CM)和 LB 培养基配制、农杆菌介导转化(*Agrobacterium tumefaciens*-mediated transformation, ATMT)和突变体筛选均参照文献[41]。大肠杆菌感受态细胞(Trans1-T1 Phage Resistant Chemically Competent Cell)购自北京全式金生物技术股份有限公司, 根癌农杆菌感受态细胞(AGL-1 Chemically Competent Cell)购自上

海唯地生物技术有限公司。本研究使用的引物由杭州有康生物科技有限公司合成, 引物见表 1。

1.2 生物信息学分析

以稻瘟病菌 *MoLCB3* (*MGG_09184*)基因的蛋白序列为检索对象, 在 NCBI (<https://www.ncbi.nlm.nih.gov/>)中检索。得到的蛋白质序列使用 ClustalW 网站 (<https://www.genome.jp/tools-bin/clustalw>)序列比对, 比对结果利用 ESPript 网站 (<https://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>)作序列比对图和导入 MEGA 11 构建系统发育树。

1.3 敲除突变体的获得

以野生型菌株 Guy11 基因组为模板, 使用引物对 *lcb3-upF/lcb3-upR* 和 *lcb3-downF/lcb3-downR* 分别扩增 *MoLCB3* 上、下游各 2.0 kb 左右的片段, 通过电泳检测片段大小并回收, 然后把上、下游片段和潮霉素抗性基因 *HPH* 片段(通过引物对 *HPH-F/HPH-R* 扩增回收)通过多片段融合酶和经酶切后的 PKO3A 载体融合。将重组载体转入大肠杆菌感受态细胞, 挑取适量单克隆菌落进行 PCR 验证(*lcb3-upF/lcb3-upR*、*lcb3-downF/lcb3-downR*、*HPH-F/HPH-R*), 能同时扩增基因上、下游片段和潮霉素片段, 则证明转入成功。确认序列准确的单菌落经扩大培养后提取质粒, $-20\text{ }^{\circ}\text{C}$ 保存备用。利用农杆菌介导转化方法, 将质粒转入农杆菌中, 随后采用潮霉素抗性筛选获得了稻瘟病菌转化子。提取所获得的敲除转化子的基因组 DNA, 用引物对 *lcb3-upyzF/HPH-yszR* 进行 PCR 检测上游和潮霉素抗性基因重组片段, 用引物对 *lcb3-innerF/lcb3-innerR* 检测基因内部片段, 若上游重组片段有, 基因内部片段无, 则证明转化子正确, 基因成功敲除。*MoLCB4* 基因敲除也是类似, 但转入片段及验证将 *HPH* 改用双丙氨磷抗性基因 *BAR*。在获得 $\Delta Molcb3$ 突变体菌株的基础上, 再敲除其 *MoLCB4* 基因, 得到 $\Delta Molcb3\Delta Molcb4$ 突变体菌株。

表 1 本研究所用引物

Table 1 Primers used in this study

| Primers name | Primer sequences (5'→3') |
|---------------|---|
| lcb3-upF | AGGCTAACTGACTCTAGAATATGTGCTATAGACAGGTCAC |
| lcb3-upR | TGTTGACCTCCACTAAAGATTGGCCCGAAAGGTG |
| lcb3-downF | GGAATAGAGTAGATGAAACACCCTTTGTTCTCTCAAC |
| lcb3-downR | CGACGGCCAGTGCCAAGCTTATCGGCATGATGCTCAACTGG |
| lcb3-upyzF | CTGGTGTAGAATGTGGCG |
| lcb3-innerF | CTGCCAAAGATTGCTGTCAATG |
| lcb3-innerR | TCCACACCAAAACATGATGGG |
| lcb4-upF | AGGCTAACTGACTCTAGACGACGCTGATGATCAGGATGGC |
| lcb4-upR | TTCAATATCATCTTCTGAAAGTTTCCGAGGGAAT |
| lcb4-downF | CCGTCACCGAGATTTAGCTTGACGTCGCTCTCGTTG |
| lcb4-downR | CGACGGCCAGTGCCAAGCTTTCGATCCGCCGCATATTGTGT |
| lcb4-upyzF | TGCAAAAAATCTCAGCGC |
| lcb4-innerF | TCAATCGTGGGAGACAAGCT |
| lcb4-innerR | GACGATCTCGAGCTCCATG |
| K5-GFP-lcb3-F | ATCAATCACAATGGCCATGAAGCGAAACAACCGAGGCT |
| K5-GFP-lcb3-R | CGCCCTTGCTCACCATCCTTGGCTGGCGTAGGTGTT |
| HPH-yzR | GCAGCAGATGATAATAATGTCC |
| BAR-yzR | TGGGGCTGATCTGACCAGTTGC |
| HPH-F | TAGTGGAGGTCAACAATGAATG |
| HPH-R | CATCTACTTATTCTTTGCC |
| BAR-F | AGAAGATGATATTGAAGGAGCA |
| BAR-R | CTAAATCTCGGTGACGGGCAGG |
| Tubulin-F | TGGAGCGTATGAGCGTCTAC |
| Tubulin-R | AAGATGGCAGAGCAGGTCAG |

1.4 *Molcb3* 基因互补菌株的获得

设计引物对 K5-GFP-lcb3-F/K5-GFP-lcb3-R, 在基因上游连接绿色荧光蛋白 (green fluorescence protein, GFP) 基因, 扩增 *MoLCB3* 基因全长片段, 采用同源重组的方法连接到含有 *BAR* 抗性筛选的载体质粒 pKD5 上。首先, 对重组质粒进行测序, 将序列正确的重组质粒转入农杆菌, 利用 ATMT 方法转入 $\Delta Molcb3$ 的孢子中, 最后得到用 *BAR* 抗性筛选的转化子。挑取转化子菌丝在绿色荧光显微镜下观察荧光, 将有荧光的转化子做上标记进一步 PCR 验证, 若扩增出 *MoLCB3* 基因片段, 则成功获得互补菌株 $\Delta Molcb3-C$ 。

1.5 菌株形态和生长速度测定

将获得的 Guy11、 $\Delta Molcb3$ 、 $\Delta Molcb3-C$ 、 $\Delta Molcb4$ 和 $\Delta Molcb3\Delta Molcb4$ 菌株在完全培养基上活化, 用灭菌刀片在菌落边缘区域切块, 选取同样大小的菌块分别接种在新的完全培养基上, 在 25 °C 下培养 7 d (光照 16 h, 黑暗 8 h)。之后用十字交叉法测量菌落生长直径, 记录、拍照保存。每个菌株设置 3 个重复, 实验重复 3 次。

1.6 产孢量、孢子类型和附着胞发育测定

每个培养皿加入 3 mL 的 ddH₂O, 分别将在完全培养基上培养 12 d 的野生型和待测菌株菌落表面的分生孢子清洗完全, 收集至 20 mL 离心管中, 7 500 r/min 离心 5 min 弃上清, 收集孢

子。最终离心管定容至 2 mL, 将孢子液滴在血球计数板上, 在显微镜下观察、统计分生孢子数目, 并记录数据, 试验重复 3 次。将孢子浓度稀释 5×10^4 个/mL 后, 滴在载玻片上用显微镜观察, 并用细胞壁钙荧光白(calcofluor white, CFW)荧光染色剂对分生孢子细胞壁和隔膜进行染色, 统计不同孢子类型数目, 每次重复统计 3 次, 重复实验 3 次。同时将上述浓度的孢子液滴至覆有疏水膜的玻片上, 28 °C 黑暗保湿培养, 在诱导 4、8、24 hpi 的时候用显微镜观察孢子萌发和附着胞形成情况。

1.7 致病性测定

剪取培育 7–8 d 生长良好的大麦叶片和三叶一芯期的水稻的第三叶, 利用打孔器对在完全培养基培养 7 d 的待测菌株进行打孔, 将大小均匀的菌丝块接种至离体大麦叶片和水稻叶片上, 保湿培养 4 d, 观察叶片发病情况并拍照记录。每个菌株设置 3 个重复, 实验重复 3 次。

1.8 高渗胁迫和细胞壁胁迫试验

以完全培养基为基底培养基, 分别添加 0.7 mol/L 氯化钾(KCl)、0.01% 十二烷基硫酸钠(sodium dodecyl sulfate, SDS)和 400 µg/mL 刚果红(Congo red, CR)。将各测试菌株接种于胁迫培养基表面, 于 28 °C 条件下暗培养 7 d 后, 测量菌落直径并统计相对生长率。每组实验独立重复 3 次, 每处理设置 3 个生物学重复。

1.9 真菌脂质合成抑制剂胁迫试验

以完全培养基为基底培养基, 分别添加 1 µg/mL 三唑酮(triadimefon, Tri)和 1 µg/mL 多球壳菌素(myriocin, Myr)。将各测试菌株接种于胁迫培养基表面, 于 28 °C 条件下暗培养 7 d 后, 测量菌株菌落直径并统计相对生长率。每组实验独立重复 3 次, 每处理设置 3 个生物学重复。

1.10 高温胁迫试验

将各待测菌株菌丝块接种于完全培养基平板上, 分别于 25 °C 和 30 °C 暗培养 7 d, 之后用十字交叉法测量菌落生长直径, 记录、拍照保存。每个菌株设置 3 个重复, 实验重复 3 次。

1.11 靶向脂质相对定量分析

将 Guy11、 $\Delta Molcb3$ 、 $\Delta Molcb4$ 和 $\Delta Molcb3\Delta Molcb4$ 菌株在 CM 液体培养基中培养 60 h, 分别收集菌丝, 每个菌株设置 3 个重复, 送至上海拜谱生物科技有限公司进行靶向脂质含量测定。对实验定量到的靶向脂质多反应监测(multiple reaction monitoring, MRM)结果进行差异代谢物表达及功能分析, 利用 GraphPad Prism 8 软件绘制筛选的脂质相对含量差异图。

2 结果与分析

2.1 MoLCB3 基因的鉴定

通过生物信息学分析, 稻瘟病菌 *MoLCB3* 基因(*MGG_09184*)位于稻瘟病菌第 I 条染色体上, 序列全长为 3 161 bp, 包含 6 个外显子, 4 个内含子, 编码包含 597 个氨基酸的蛋白质, 对序列跨膜结构分析发现, 其有 6 个跨膜区。与稻瘟病菌单基因编码不同, 模式真菌酿酒酵母中有 2 个同源基因编码 S1PP, 分别是 *LCB3* 和 *YSR3*, 这 2 种蛋白具有 53.00% 的一致性, *Lcb3* 负责大多数细胞活动^[33,40]。通过同源对比发现, *MoLcb3* 蛋白与酿酒酵母中 *Lcb3* 蛋白和 *Ysr3* 蛋白的氨基酸一致性分别达到 29.58% 和 28.96%。进一步与其他真菌的同源蛋白进行系统发育树分析, 其中稻瘟病菌 *MoLcb3* 与小麦全蚀病菌(*Gaeumannomyces tritici*) R3-111a-1 和炭角菌(*Xylariaceae* sp.) FL0016 同源蛋白的遗传关系较近(图 1), 氨基酸一致性分别为 71.19% 和 65.57%。

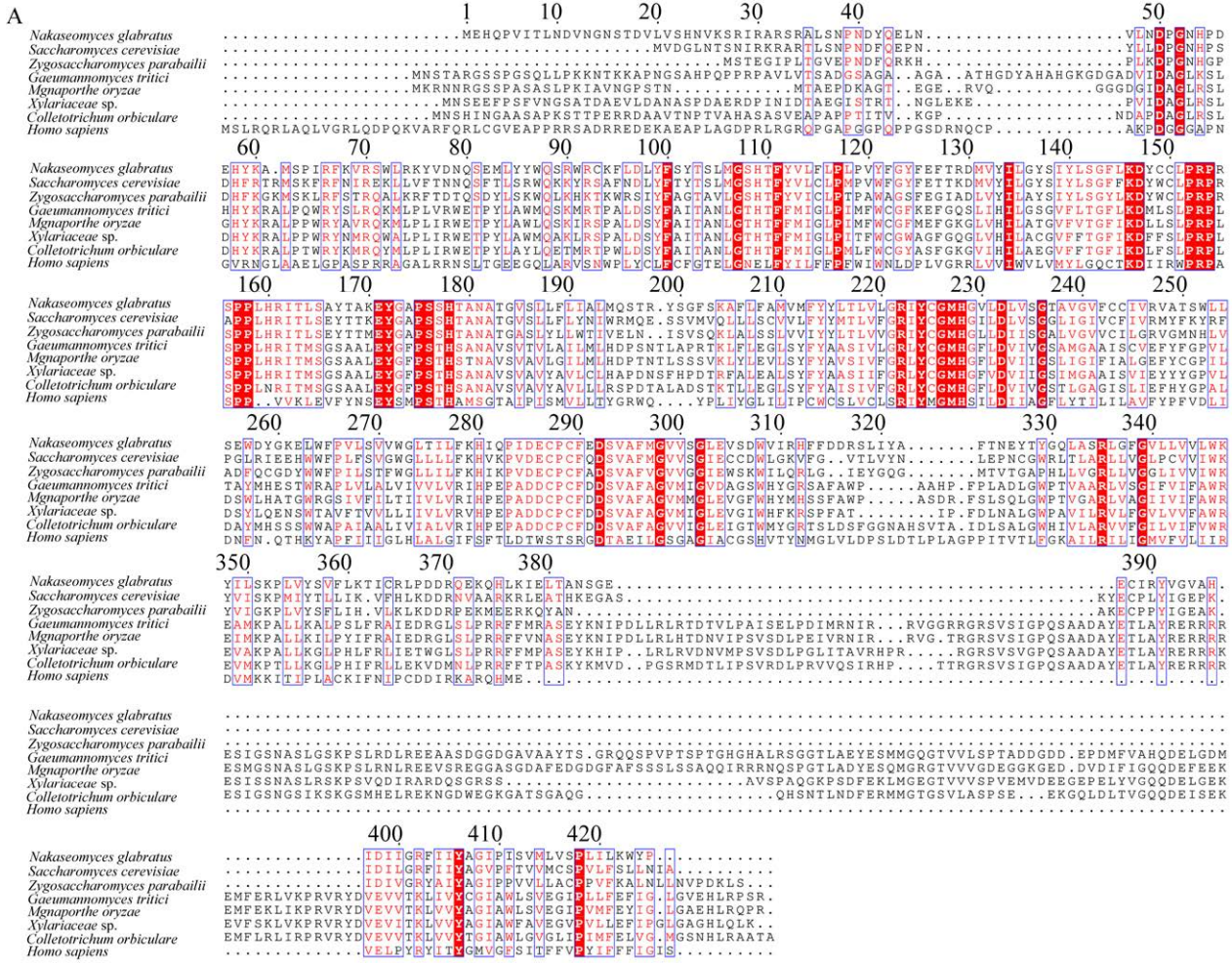


图 1 MoLcb3 同源基因序列比对与系统发育分析

Figure 1 Sequences comparison and phylogenetic analysis of MoLcb3 homologous gene. A: Protein sequences alignment of homologous genes in *Magnaporthe oryzae* MoLcb3 (KAI7921492.1), *Saccharomyces cerevisiae* Lcb3 (KZV10140.1), *Gaeumannomyces tritici* (XP_009230231.1), *Xylariaceae* sp. (KAI1337660.1), *Colletotrichum orbiculare* (TDZ17510.1), *Zygosaccharomyces parabolii* (AQZ17239.1), *Nakaseomyces glabratus* (KTB14151.1), *Homo sapiens* (NP_110418.1), the red background and purple box indicated the identity of all and more than 6 conserved nucleotides. B: Based on the sequence comparison results, MEGA 11 program was used to construct the phylogenetic tree of Lcb3 genes.

2.2 Δ Molcb3 突变体的获得

根据 DNA 同源重组的原理将 *MoLCB3* 基因替换成潮霉素抗性基因, 以达到敲除的目的(图 2A), 并经过潮霉素抗性筛选得到转化子。用目的基因外部引物对 lcb3-upyzF/HPH-yszR 验证转化子目的基因是否成功被替换, 图 2B 显示泳道 3-8 扩增到 2 000 bp 的条带, 对照泳道 2 未扩增出条带, 同时用目的基因内部引物对 lcb3-innerF/lcb3-innerR 进一步验证, 图 2C 显示

仅泳道 2 扩增到约 500 bp 的条带(部分 *MoLCB3* 基因), 用对照引物对 Tubulin-F/Tubulin-R 都能扩增出 1 000 bp 的条带, 说明泳道 3-8 的相应菌株为正确的 *MoLCB3* 基因敲除突变体菌株 (Δ Molcb3)。

2.3 MoLcb3 参与稻瘟病菌营养生长与产孢

在 CM 板上生长 7 d 后观察菌落形态, 发现稻瘟病菌野生型 Guy11 气生菌丝浓密, 并呈灰绿色, 然而 Δ Molcb3 突变体的菌落菌丝稀疏,

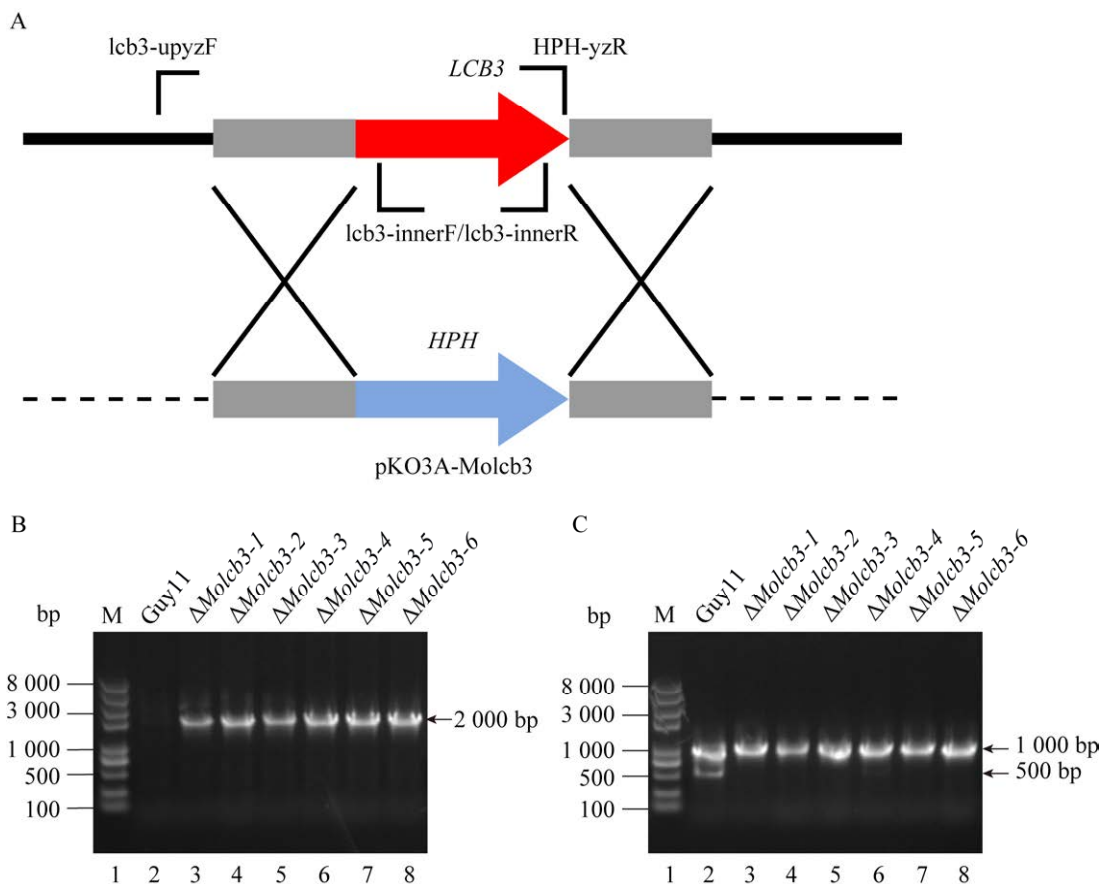


图 2 *MoLCB3* 基因敲除示意图及 Δ Molcb3 突变体替换基因内外部双重 PCR 验证

Figure 2 *MoLCB3* gene knockout diagram and internal and external PCR validation of Δ Molcb3 mutant replacement gene. A: Knockout vector pKO3A-Molcb3 and *MoLCB3* target gene knockout. The red arrow represents the target gene *MoLCB3*, the blue arrow represents the *HPH* gene, the position of the primer is marked accordingly. B: 2 000 bp external fragment of *MoLCB3* gene amplified with lcb3-upyzF/HPH-yszR primer. C: lcb3-innerF/lcb3-innerR primers were used to further verify the deletion of targeted genes in different strains, and bands of about 500 bp and 1 000 bp corresponded to *Molcb3* and β -tubulin genes, respectively. M: DNA marker.

气生菌丝呈现黄白色，互补菌株 $\Delta Molcb3-C$ 的菌落形态与 Guy11 类似(图 3A)，二者的菌落直径分别为 (4.68 ± 0.05) cm、 (4.70 ± 0.01) cm，而 $\Delta Molcb3$ 突变体的菌落直径仅有 (3.85 ± 0.02) cm (图 3B)。表明 MoLcb3 的缺失影响了稻瘟病菌的菌落形态和生长速度。产孢量统计结果表明， $\Delta Molcb3$ 突变体的分生孢子极少，而 Guy11 和互补菌株的产孢量正常(图 3C)。

2.4 MoLcb3 影响孢子形态和附着胞形成

为了探究 MoLCB3 基因除了对稻瘟病菌产孢数目的影响外，是否还对其分生孢子其他功能产生作用，经显微镜下观察分生孢子发现， $\Delta Molcb3$ 突变体孢子无隔和单隔孢子占比较高，

而正常的孢子往往是两隔的(图 4A)。为了更清晰地对分生孢子进行观察和统计，用荧光染料 CFW 对各菌株分生孢子进行染色，在紫外光下观察分生孢子。经统计， $\Delta Molcb3$ 突变体分生孢子相较于其他菌株畸形率更高(图 4B)，表明 MoLcb3 参与了分生孢子的分隔形成过程。将分生孢子悬浮液置于疏水玻片表面诱导附着胞形成，发现 $\Delta Molcb3$ 突变体虽然孢子的畸形率较高，但不同类型孢子还是可以正常萌发。当 $\Delta Molcb3$ 突变体的单隔孢子在诱导 4 h 时，相较于其他类型孢子，芽管顶管未形成初始附着胞(图 4C)，结果说明 MoLcb3 缺失影响孢子畸形的同时，还影响其附着胞的初期形成。

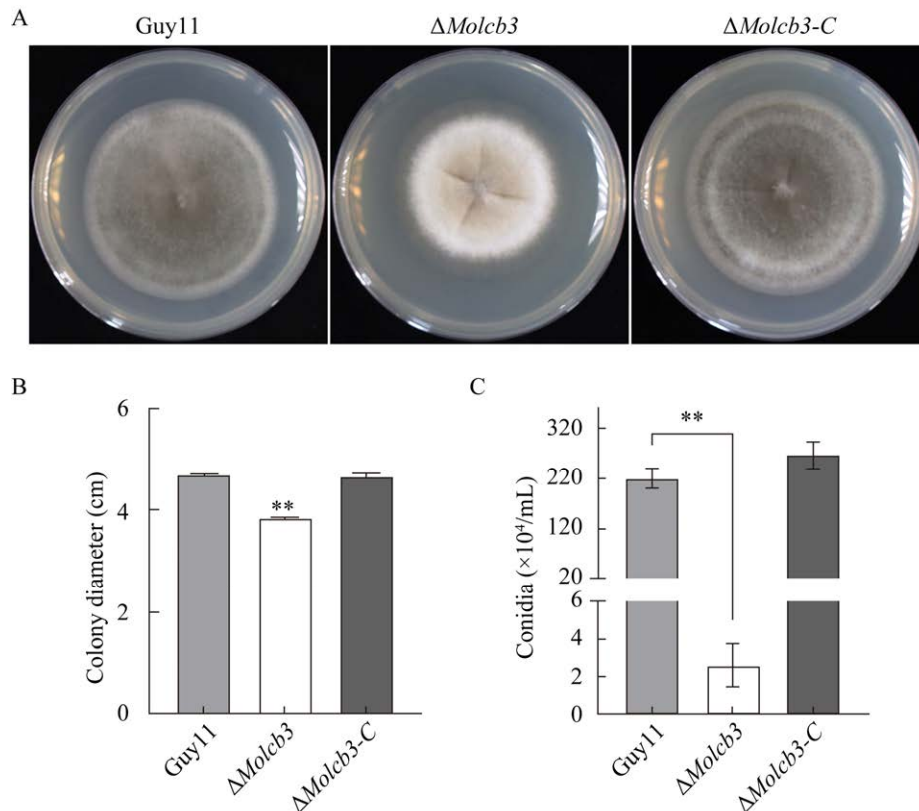


图 3 $\Delta Molcb3$ 的菌落形态、生长速度和产孢情况

Figure 3 Colony morphology, growth rate and sporulation of $\Delta Molcb3$. A: Colony morphology of wild type Guy11, $\Delta Molcb3$ mutant and replacement strain $\Delta Molcb3-C$ cultured at 25 °C on CM medium for 7 days. B: The colony diameters of wild type Guy11, $\Delta Molcb3$ and complementary strain $\Delta Molcb3-C$ on CM medium were statistically analyzed. C: An asterisk indicates a significant difference (** $P < 0.01$).

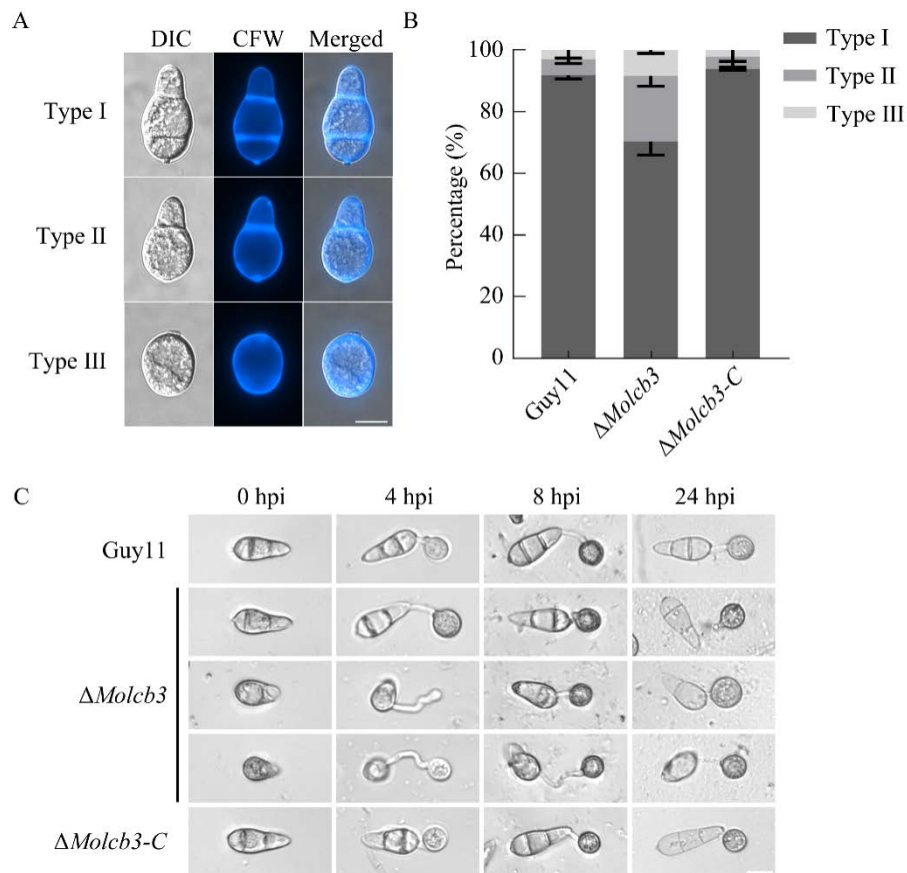


图 4 Δ Molcb3 的分生孢子形态和附着胞形成

Figure 4 Conidial morphology and appressoria formation of Δ Molcb3. A: The morphology of Δ Molcb3 mutant conidia was observed and the cell wall and diaphragm of conidia were stained with Calcofluor white (CFW). Scale=10 μ m. Type I: Phragmoconidium; Type II: Didymoconidium; Type III: Ameroconidium. B: The abnormal rate of conidia in each strain was calculated. Type I: Phragmoconidium; Type II: Didymoconidium; Type III: Ameroconidium. C: The appressorium formation rate of wild type Guy11, Δ Molcb3 mutant and the complementary strain Δ Molcb3-C was induced on the surface of the hydrophobic membrane. Scale=10 μ m.

2.5 Δ Molcb3 突变体致病性分析

为了探究 MoLcb3 是否参与稻瘟病菌的致病过程, 采用大麦作为寄主进行致病力测定。在接种菌株 4 d 后观察发现, Guy11 和互补菌株均表现出极强的致病性, 在叶片上产生明显黄褐色扩展病斑, 而 Δ Molcb3 突变体无病斑产生, 致病性显著降低(图 5)。以上结果表明 MoLcb3 是稻瘟病菌致病过程所必需的。

2.6 Δ Molcb3 响应高渗胁迫和细胞壁胁迫

面对生长过程中遭遇的各种胁迫压力, 长期的自然选择下真菌已经形成了良好的抗胁迫能



图 5 Δ Molcb3 在大麦上的发病情况检测

Figure 5 Pathogenicity detection on barley leaves. Mycelium plugs of wild type Guy11, Δ Molcb3 mutant, and the complementary strain Δ Molcb3-C were placed on the isolated leaves of 7-day-old barley and photographed 4 days later.

力^[42-43]，稻瘟病菌当然也不例外。为了验证 *MoLcb3* 对稻瘟病菌抗胁迫能力的影响，本研究通过添加外界环境胁迫因子来检测突变体在高渗胁迫和细胞壁胁迫下的生长情况。将 *Guy11*、 Δ *Molcb3* 突变体和 Δ *Molcb3-C* 互补菌株分别接种在含 0.7 mol/L KCl、0.01% SDS 和 400 μ g/mL CR 的完全培养基平板上，7 d 后测量菌落直径并计算其相对生长率。结果显示，*Guy11* 在含 KCl 的相对生长率为(67.7 \pm 1.0)%， Δ *Molcb3* 突变体在含 KCl 的相对生长率为(73.2 \pm 0.8)%，高渗胁迫压力对突变体的抑制效果显著减弱(图 6)，说明稻瘟病菌 *MoLCB3* 基因缺失后，对高渗胁迫更加耐受。*Guy11* 在含 SDS、CR 的相对生长率分别为(51.1 \pm 0.7)%、(51.1 \pm 1.7)%， Δ *Molcb3* 突变体分别为(57.7 \pm 1.0)%、(42.5 \pm 2.8)%，表现为 SDS 对突变体的抑制效果减弱(图 6)，而突变体对 CR 更加敏感。虽然 SDS 和 CR 都能够干扰细胞壁完整性，但其作用机理不一致，CR 会与几丁质和 β -1,3-葡聚糖形成复合物，干扰细胞壁组装^[44-45]，SDS

渗透细胞膜以激活应激反应^[46]，但色氨酸可以提高酿酒酵母对 SDS 的抵抗力，却对 CR 无效果^[47]。

2.7 Δ *Molcb3* 对真菌脂质合成抑制剂的敏感情况

为了进一步探究 *Molcb3* 基因对脂质合成代谢通路是否有影响，本研究测试了菌株对 2 种真菌脂质合成抑制剂的敏感情况。将 *Guy11*、 Δ *Molcb3* 突变体和 Δ *Molcb3-C* 互补菌株分别接种在含添加 1 μ g/mL 三唑酮(麦角甾醇合成抑制剂，通过抑制真菌麦角甾醇生物合成途径去甲基化酶的活性减少麦角甾醇的合成，从而破坏真菌的细胞膜^[48-49])和 1 μ g/mL 多球壳菌素(丝氨酸-棕榈酰转移酶抑制剂，抑制鞘脂的从头合成)的完全培养基平板上，7 d 后测量菌落直径并计算其相对生长率。结果显示，*Guy11* 在含 Tri 的相对生长率为(77.5 \pm 1.0)%， Δ *Molcb3* 突变体为(81.6 \pm 1.0)%，抑制效果显著减弱(图 7)。*Guy11* 在含 Myr 的相对生长率为(67.7 \pm 2.5)%， Δ *Molcb3* 突变体为(62.0 \pm 1.9)%，显著低于野生型(图 7)。

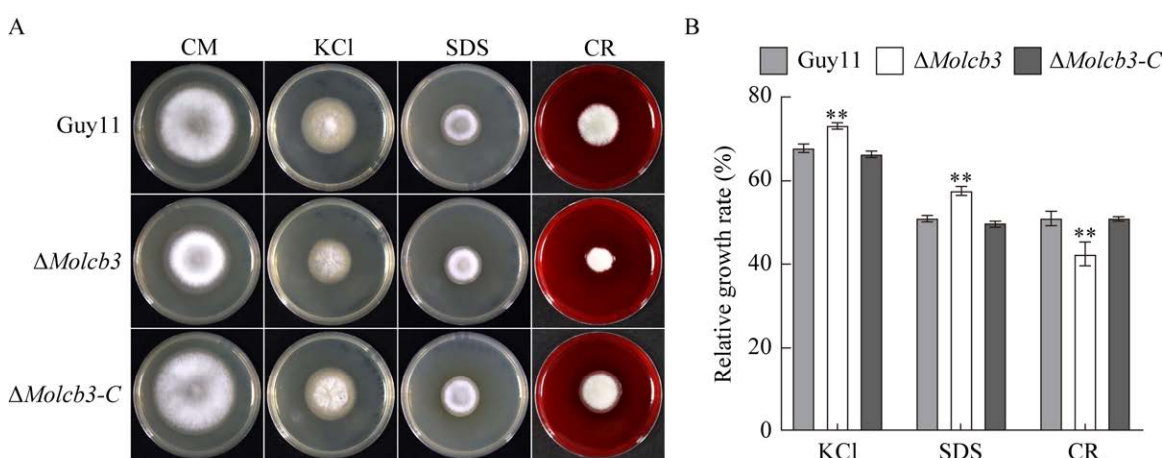


图 6 各菌株在含高渗胁迫和细胞壁胁迫因子条件下的生长情况

Figure 6 The growth of each strain under hypertonic stress and cell wall stress factors. A: Hypertonic stress agent KCl, cell wall stress agent SDS and Congo red (CR) were added to CM substrate. After 7 days of cultivation under dark conditions, the growth of the strain was observed. B: Colony diameters of wild type *Guy11*, Δ *Molcb3* mutant and complementary strain Δ *Molcb3-C* on stress medium were statistically analyzed. Asterisks indicate significant differences (**: $P < 0.01$).

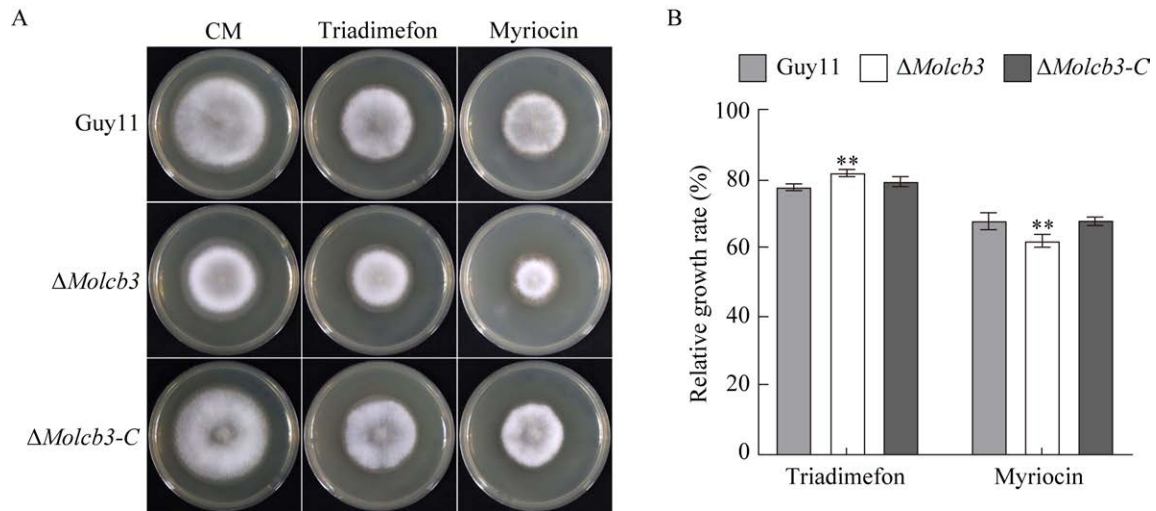


图 7 各菌株在含两种真菌脂质合成抑制剂条件下的生长情况

Figure 7 The growth of each strain under the condition of two fungal lipid synthesis inhibitors. A: The fungal lipid synthesis inhibitors triadimefon and myriocin were added on CM substrate, respectively. After 7 days of cultivation under dark conditions, the growth of the strain was observed. B: Colony diameters of wild type Guy11, $\Delta Molcb3$ mutant and complementary strain $\Delta Molcb3-C$ on stress medium were statistically analyzed. Asterisks indicate significant differences (**: $P < 0.01$).

2.8 $\Delta Molcb3$ 对高温胁迫的敏感情况

真核细胞对温度升高具有高度保守的反应，称为热休克反应^[50]。酵母和哺乳动物细胞热休克会引发 SPT 的急性激活，导致鞘脂的从头生物合成^[51-52]，鞘脂是参与真核生物热休克反应许多方面的生物活性信号分子^[53-55]。本研

究测试了 $\Delta Molcb3$ 突变体在高温胁迫下的生长情况，发现 Guy11 在 30 °C 的相对生长率为 (53.7±1.2)%， $\Delta Molcb3$ 突变体为 (46.4±1.3)%，高温胁迫对于突变体的抑制效果明显增强(图 8)，结果说明 MoLcb3 在稻瘟病菌热胁迫应激过程中发挥着功能。

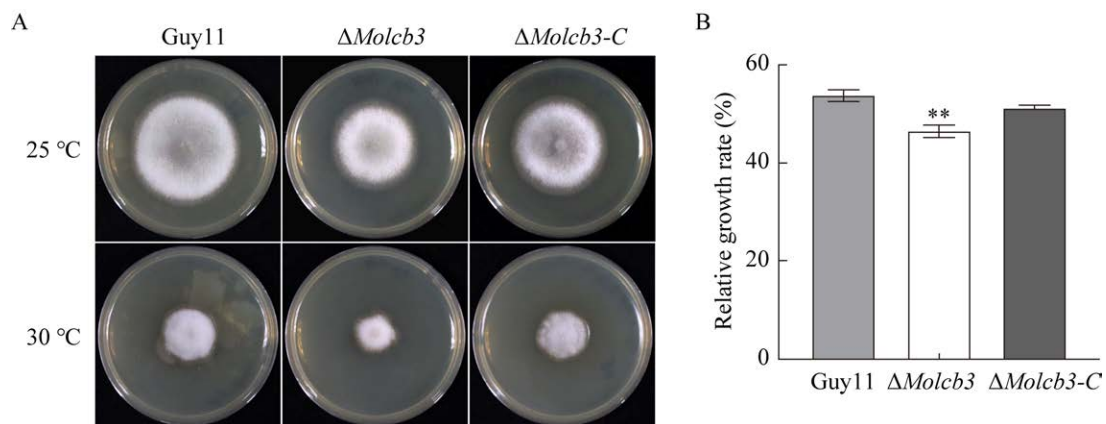


图 8 各菌株在高温胁迫下的生长情况

Figure 8 The growth of each strain under high temperature stress. A: The colony morphology of wild type Guy11, $\Delta Molcb3$ mutant and the complementary strain $\Delta Molcb3-C$ were cultured on CM medium at 25 °C and 30 °C for 7 days. B: The relative growth rate of wild type Guy11, $\Delta Molcb3$ mutant and complementary strain $\Delta Molcb3-C$ were statistically analyzed at 30 °C temperature. Asterisks indicate significant differences (**: $P < 0.01$).

2.9 敲除 *MoLCB4* 能缓解 *MoLcb3* 缺失带来的影响

在鞘脂生物学中,神经酰胺和鞘氨醇以及鞘氨醇-1-磷酸在多种细胞中发挥拮抗作用^[56]。Cer 和 Sph 可以介导细胞凋亡、细胞周期阻滞和细胞分化,而 S1P 则促进细胞增殖、存活和抑制凋亡,这有点像是“鞘脂变阻器”,特别是在对压力源或生长刺激的反应中,这些简单鞘脂的相对水平是细胞存活的重要决定因素^[57]。Sph、DHS 和 PHS 可以经鞘氨醇激酶磷酸化是一个可逆的动态过程^[58]。磷酸化和去磷酸化常常是激活关键调控蛋白和控制信号通路传导的开关,一旦磷酸化过程发生异常,相关信号通路会出现功能失调^[59]。

为了进一步探究磷酸酶 *MoLcb3* 和激酶

MoLcb4 之间的关系。在 CM 板上生长 7 d 后观察菌落形态,发现 $\Delta Molcb4$ 突变体的菌落形态与 Guy11 类似, $\Delta Molcb3\Delta Molcb4$ 突变体的菌落相较于 $\Delta Molcb3$ 突变体,部分恢复灰绿色(图 9A)。通过测量直径发现, Guy11 的菌落直径分别为 (4.55 ± 0.04) cm,而 $\Delta Molcb3$ 突变体的菌落直径仅有 (3.73 ± 0.03) cm, $\Delta Molcb4$ 突变体和双敲突变体 $\Delta Molcb3\Delta Molcb4$ 菌落直径介于两者之间,分别为 (4.37 ± 0.03) cm 和 (4.10 ± 0.03) cm (图 9B)。表明 *MoLcb3* 和 *MoLcb4* 缺失都会影响稻瘟病菌的菌落形态和生长速度,但当 *MoLcb3* 和 *MoLcb4* 同时缺失时,对比 $\Delta Molcb3$ 突变体菌落形态和直径大小反而有一定程度恢复。进一步统计产孢量,结果发现 $\Delta Molcb4$ 突变体和双敲突变体 $\Delta Molcb3\Delta Molcb4$ 产孢量趋势与菌落表现基本一致。

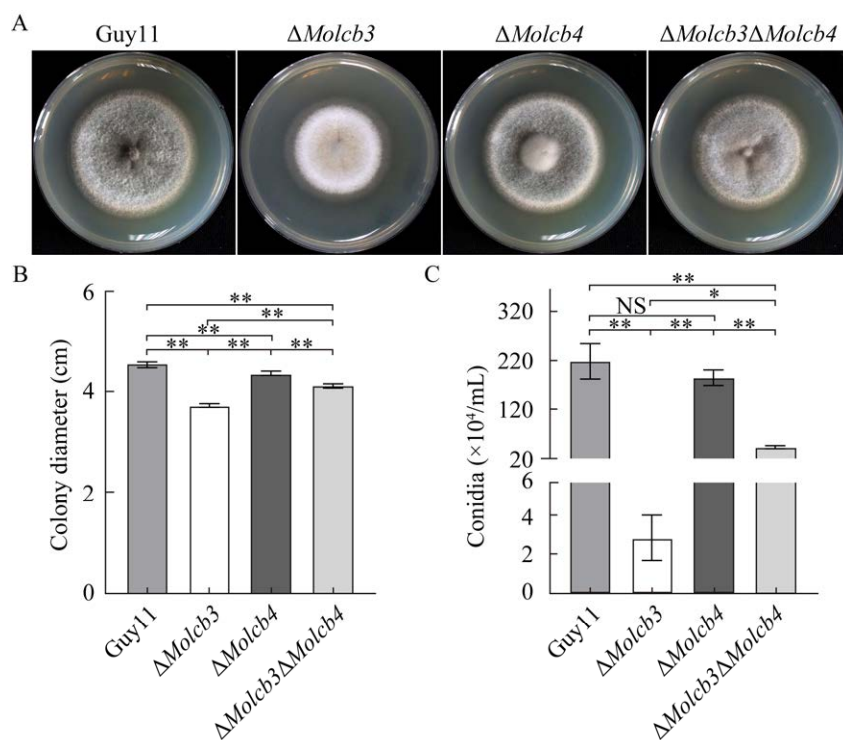


图 9 $\Delta Molcb3\Delta Molcb4$ 双敲突变体的菌落形态、生长速度和产孢情况

Figure 9 Colony morphology, growth rate and sporulation of $\Delta Molcb3\Delta Molcb4$ double-knock mutant. A: The colony morphology of wild type Guy11, $\Delta Molcb3$ mutant, $\Delta Molcb4$ mutant and $\Delta Molcb3\Delta Molcb4$ double-knock mutant were cultured on CM medium at 25 °C for 7 days. B: Colony diameters of wild type Guy11, $\Delta Molcb3$ mutants, $\Delta Molcb4$ mutants and $\Delta Molcb3\Delta Molcb4$ double-knock mutants on CM medium were statistically analyzed. The strain growing for 7 days was used for sporulation analysis. An asterisk indicates a significant difference (*: $P < 0.05$; **: $P < 0.01$), and NS indicates no significant difference.

在诱导形成附着胞试验中, $\Delta Molcb4$ 突变体和 $\Delta Molcb3\Delta Molcb4$ 双敲突变体附着胞都能够正常形成(图 10A)。在大麦和水稻作为寄主的致病力测定试验中, 在接种菌株 4 d 后观察发现, 不管是大麦叶片还是水稻叶片, Guy11 和 $\Delta Molcb4$ 突变体均表现出极强的致病性, 在叶片上产生明显黄褐色扩展病斑, 而 $\Delta Molcb3$ 突变体几乎丧失致病性, $\Delta Molcb3\Delta Molcb4$ 双敲突变体能够恢复一定致病性(图 10B、10C)。以上结果表

明 MoLcb4 的缺失能解除 MoLcb3 缺失对致病力的影响。

对 $\Delta Molcb4$ 突变体和 $\Delta Molcb3\Delta Molcb4$ 双敲突变体在各类胁迫下的敏感情况同样进行了检测, 结果发现在 KCl、SDS 和 CR 的胁迫下, $\Delta Molcb3\Delta Molcb4$ 双敲突变体相对生长率恢复至野生型同一水平, 说明 MoLcb4 缺失能够缓解 MoLcb3 缺失所引起的高渗胁迫和细胞壁完整性胁迫敏感。在 Tri 和 Myr 的胁迫下, $\Delta Molcb3$

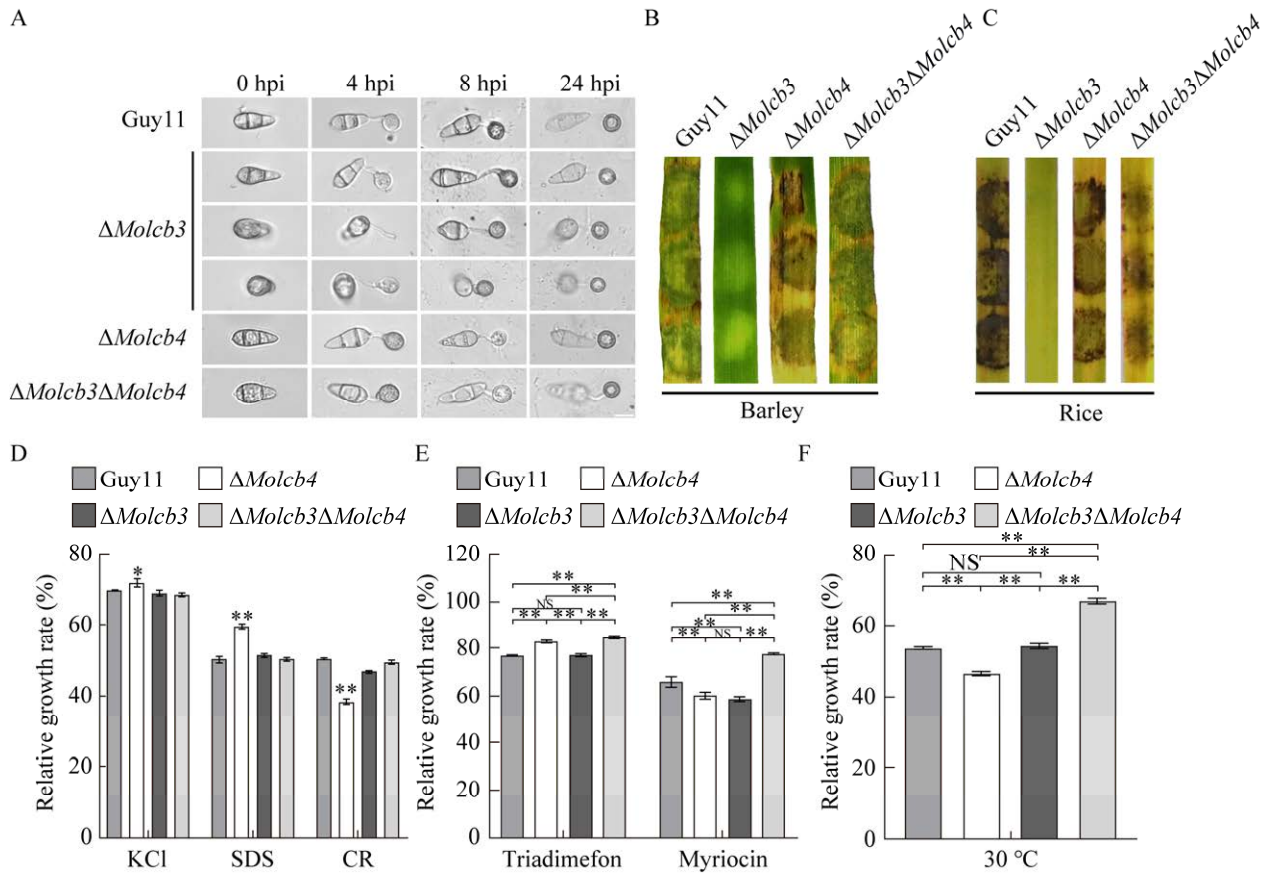


图 10 $\Delta Molcb3\Delta Molcb4$ 在水稻和大麦上的发病情况

Figure 10 Incidence of $\Delta Molcb3\Delta Molcb4$ in rice and barley. A: The appressoria formation rate of the wild type Guy11, $\Delta Molcb3$, $\Delta Molcb4$, and $\Delta Molcb3\Delta Molcb4$ strains were induced on the surface of the hydrophobic membrane. Scale=10 μ m. B: Pathogenicity detection on barley leaves. Mycelial plugs of the wild type Guy11, $\Delta Molcb3$, $\Delta Molcb4$, and $\Delta Molcb3\Delta Molcb4$ were placed on the isolated leaves on 7-day-old barley and photographed at 4 days. C: The same mycelium pieces were inoculated on isolated rice leaves growing at 14 days and photographed at 4 days. D, E, F: The growth rates of $\Delta Molcb3$, $\Delta Molcb4$ and the $\Delta Molcb3\Delta Molcb4$ double-knock mutants at KCl, SDS, CR, Tri, Myr and heat stress conditions, respectively. An asterisk indicates a significant difference (*: $P < 0.05$; **: $P < 0.01$), and NS indicates no significant difference.

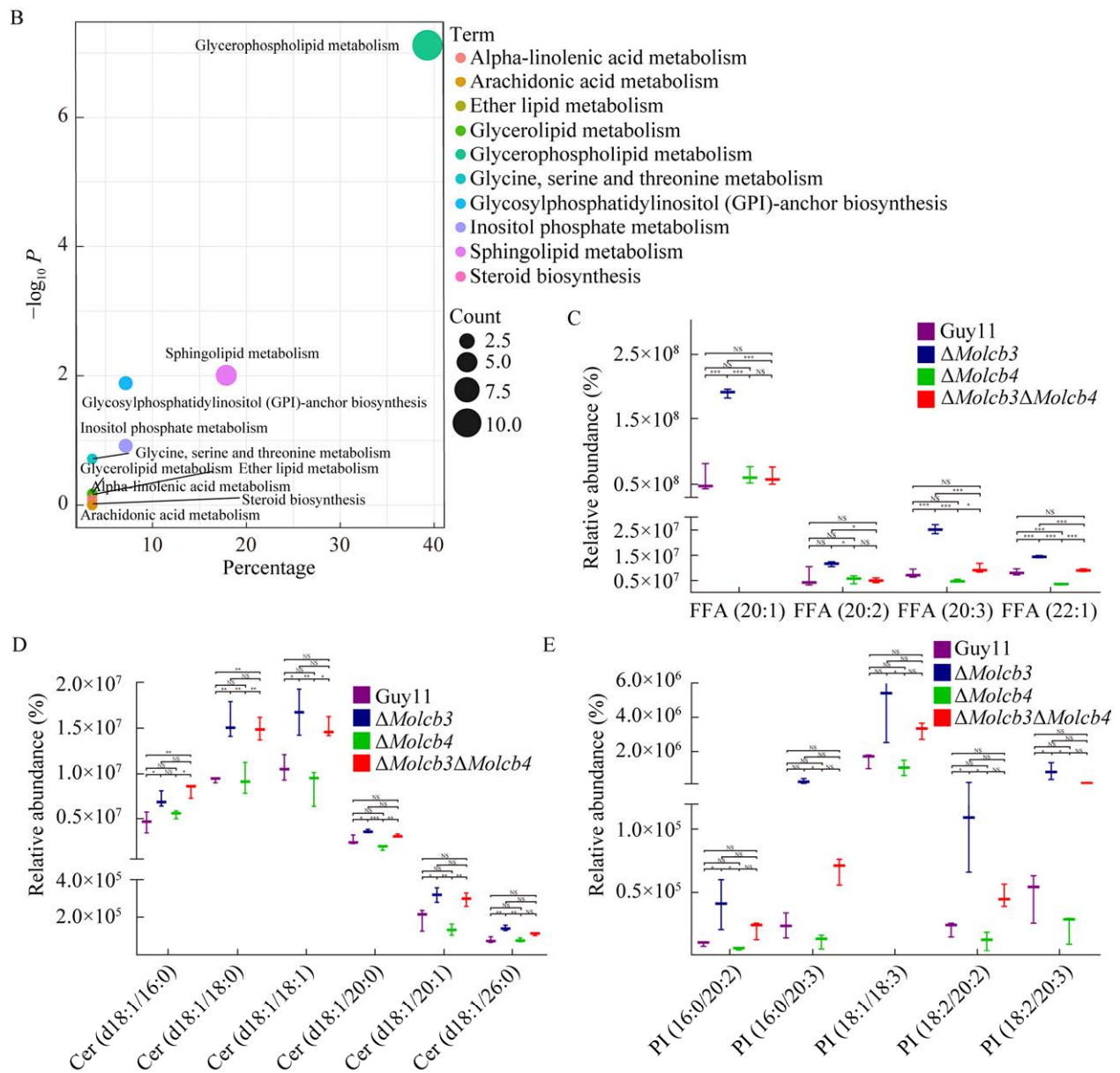


图 11 利用脂质组学分析各菌株脂质含量

Figure 11 Lipidomics was used to analyze the lipid content of each strain. A: Cluster heat maps, with significant differences in lipid content between groups (higher in red, lower in blue). B: KEGG enrichment analysis showed that there were significant differences in lipids involved in lipid metabolic pathways such as glycerophospholipid metabolism, sphingolipid metabolism, and glycosylphosphatidylinositol (GPI)-anchored biosynthesis. C: The relative content of FFA. D: The relative content of Cer. E: The relative content of PI. An asterisk indicates a significant difference (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$), and NS indicates no significant difference.

磷脂酰乙醇胺(phosphatidylethanolamines, PE)、甘油三酯(triacylglycerols, TAG)、神经酰胺、磷脂酰肌醇(phosphatidylglycerols, PI)等异常调节的

脂质参与了甘油磷脂代谢、鞘脂代谢和糖基磷脂酰肌醇(glycosylphosphatidyl inositol, GPI)-锚定生物合成等过程(图 11B)。其中发现 $\Delta Molcb3$ 突

变体中游离脂肪酸(free fatty acid, FFA) FFA (20:1)、FFA (20:2)、FFA (20:3)、FFA (22:1)含量相对于其他菌株显著升高(图 11C)。 $\Delta Molcb3$ 突变体和 $\Delta Molcb3\Delta Molcb4$ 双敲突变体中 Cer (d18:1/16:0)、Cer (d18:1/18:0)、Cer (d18:1/18:1)、Cer (d18:1/20:0)、Cer (d18:1/20:1) 和 Cer (d18:1/26:0)含量相对于野生型和 $\Delta Molcb4$ 突变体显著升高(图 11D)。PI (16:0/20:2)、PI (16:0/20:3)、PI (18:1/18:3)、PI (18:2/20:2)和 PI (18:2/20:3)也与 Cer 情况相似,都是 $\Delta Molcb3$ 突变体和 $\Delta Molcb3\Delta Molcb4$ 双敲突变体相对于野生型和 $\Delta Molcb4$ 突变体含量显著升高(图 11E)。这些结果进一步证实了 MoLcb3 的缺失会影响菌株内脂质稳态,从而涉及其他脂质代谢途径。

3 讨论与结论

鞘脂代谢是真核生物界的一个多分支途径,途径的基本结构在整个进化过程中是保守的,但一些细节在不同物种之间有所不同^[6,60]。几种鞘脂代谢物,特别是神经酰胺、鞘氨醇和鞘氨醇-磷酸作为控制细胞生长和细胞程序性死亡的关键生物活性分子,某类鞘脂的扰动可能增强或干扰其他类型鞘脂的作用,因此,鞘脂水平受到特定生物合成和分解代谢途径的严格调节^[61-62]。在拟南芥(*Arabidopsis thaliana*)保护细胞中负责 S1P 生成的 SK 会受到植物激素脱落酸的刺激, S1P 再调节保护细胞膨胀,从而调节气孔开度^[63]。在酿酒酵母中, Lcb3 是将外源 LCBs 并入鞘脂所必需的^[64], Lcb3 的缺失会导致 LCBP 的积累和 Cer 水平降低^[40]。由于 S1P 的重要性, S1P 在医学上得到了广泛的研究,其在癌症发展、心血管功能和大脑健康等具有重要作用^[65-67]。本研究发现,稻瘟病菌 MoLcb3 蛋白与酿酒酵母 LCB3 同源,并在菌丝生长、产孢量、孢子形态和致病性等方面均起重要作用,这些研究结果对

进一步探明 MoLcb3 调控稻瘟病菌鞘脂代谢的基因网络及分子机制具有重要意义。

本研究发现, MoLcb3 缺失会导致稻瘟病菌生长减缓,产孢量显著下降,菌落直径仅为野生型 Guy11 的 81.91% (图 3B),产孢量仅为野生型 Guy11 的 1.18% (图 3C)。此外, $\Delta Molcb3$ 突变体的分生孢子畸形率显著升高,但不影响其形成附着胞(图 4B、4C), $\Delta Molcb3$ 突变体完全丧失了对大麦和水稻寄主的致病性,而 MoLcb4 的缺失不影响稻瘟病菌致病性,双敲突变体恢复一定致病性(图 10B、10C),说明 MoLcb3 在调控稻瘟病菌正常孢子形成和致病过程中可能比 MoLcb4 起着更重要的作用。

根据 $\Delta Molcb3$ 突变体在含 KCl 培养基上的生长情况,推测是 LCBP 物质含量的积累提高了稻瘟病菌对高渗胁迫的耐受性。面对 SDS 和 CR 胁迫时的相反表现,说明 MoLcb3 参与细胞完整性通路功能并不是完全单向的。另外 *MoLCB3* 基因的缺失使稻瘟病菌对 Tri 更加耐受,可能是某类脂质代谢途径的波动缓解了麦角甾醇被结合的影响。当鞘脂从头合成途径第一步关键酶 SPT 受到 Myr 抑制时, $\Delta Molcb3$ 突变体表现为更加敏感。Sph 除了从头合成,也可以通过 S1P 去磷酸化得到,可以一定程度弥补 Sph 上游合成不足,当该补救途径也受到阻断时,稻瘟病菌受到抑制是合理的。在高温胁迫下, $\Delta Molcb3$ 突变体生长显著减缓,说明 MoLcb3 在稻瘟病菌热胁迫应激过程中发挥着功能。

鞘氨醇-1-磷酸是一种多效性脂质介质,与人类各类癌症的生长、进展、转化和转移的过程有关^[68-70],针对唯一能限速催化鞘氨醇磷酸化形成 S1P 的激酶-鞘氨醇激酶,开发出了各类鞘氨醇激酶抑制剂,以发挥抗癌的功效^[71-73]。与人类不同的是,在酿酒酵母中 *LCB4* 和 *LCB5* (均编码鞘氨醇激酶)缺失或者 *LCB3* 缺失都不影响其在

培养基中正常生长^[53,58,74]。同样是真菌的稻瘟病菌, 本研究结果也表明 MoLcb4 缺失似乎不影响稻瘟病菌的正常功能, 但是 MoLcb3 缺失是会受到影响的, 此外, 敲除 *MoLCB4* 能缓解 MoLcb3 缺失带来的影响。

另外, 本研究通过脂质代谢组学分析, 鉴定到了一些可能受 MoLcb3 调控的脂质代谢途径, 如甘油磷脂代谢、鞘脂代谢、糖基磷脂酰肌醇(GPI)-锚定生物合成等, $\Delta MoLcb3$ 突变体还有多种游离脂肪酸(FFA)、神经酰胺(Cer)和磷脂酰肌醇(PI)含量较其他菌株显著升高, 含量水平基本处于 $\Delta MoLcb3$ 突变体最高, $\Delta MoLcb3\Delta MoLcb4$ 双敲突变体次之, 野生型 Guy11 再次之, $\Delta MoLcb4$ 突变体最低。说明 MoLcb3 的缺失导致这几类物质不能被很好地消耗或者合成更复杂的鞘脂, MoLcb3 直接或者间接调控稻瘟病菌 FFA、Cer 和 PI 的胞内水平。然而, 对 MoLcb3 如何调控这些基因的表达尚不清楚。

直接靶向 LCBP 的酶除了鞘氨醇-1-磷酸磷酸酶, 还有鞘氨醇-1-磷酸裂解酶, SIP 通过 SPL 裂解生成磷酸乙醇胺和十六醛类, 这一过程不可逆^[75]。在拟南芥中 *DPL1* (编码 SPL) 的较高表达显著降低了伏马菌素 B 诱导的 LCBP 和 LCB 水平升高, 并使植物对这种真菌毒素具有更强的抵抗力^[76-77]。在酿酒酵母中, 缺乏 SPT 和类黏蛋白(orosomuroid, ORM)调控的酵母突变体需要 *DPL1* 将 LCB 维持在亚致死水平^[78]。酿酒酵母中 *DPL1* 和 *YSR2* 的双突变体是致死的, 而 *DPL1*、*YSR2* 和 *LCB4* 的三重突变体是可以存活的^[79]。然而, 稻瘟病菌中 MoLcb3、MoLcb4 和 MoDpl 三者之间存在什么样的关系, 我们尚不清楚, 需要进一步研究。

综上所述, 本研究对稻瘟病菌鞘氨醇-1-磷酸磷酸酶 MoLcb3 的生物学功能进行了研究, 阐明了 MoLcb3 在菌丝生长、产孢、孢子萌发、致

病性、胁迫应激反应和维持脂质稳态等过程中起重要作用, 敲除 *MoLCB4* 能缓解 MoLcb3 缺失带来的影响。

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