水稻水通道蛋白OsPIP1;3与白叶枯病菌harpin蛋白Hpa1互作关系研究

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摘要: 本文通过片段缺失和定点突变的方法构建产生水稻(*Oryza sativa*)水通道蛋白OsPIP1;3的变异本。通过膜酵母双杂交进行蛋白互作分析, 实验结果表明水稻白叶枯病菌(*Xanthomonas oryzae* pv. *oryzae*) harpin蛋白Hpa1能与OsPIP1;3互作, 但如果将OsPIP1;3的一个胞外序列(loop E)及OsPIP1;3的第六个跨膜区域(TM6)删除, 则互作不能发生。进一步对276~279 aa进行替换, OsPIP1;3与Hpa1不再发生互作。这些结果说明, OsPIP1;3序列上的loop E和TM6上的氨基酸对OsPIP1;3–Hpa1互作有重要影响, 而276~279 aa是可能的互作位点。

关键词: Hpa1; OsPIP1;3; 定点突变; 膜酵母双杂交; 蛋白互作

水通道蛋白(aquaporin, AQP)是指细胞膜上能高效转运水分子的膜内在蛋白, 属于major intrinsic protein (MIP)超家族, 分子质量为21~31 kDa, 在动物、植物以及微生物中都广泛存在(Borgnia等1999)。根据同源性分析以及细胞定位, 植物AQP被分为4大类: 质膜内在蛋白(plasma membrane intrinsic proteins, PIPs)、液泡膜内在蛋白(tonoplast intrinsic proteins, TIPs)、Nod26膜内在蛋白(nodulin 26–like intrinsic proteins, NIPs)以及定位于内质网的小分子碱性膜内在蛋白(small and basic intrinsic proteins, SIPs) (Maurel等2008)。PIPs都含有6个跨膜结构域, 能够嵌入到细胞质膜中, 在植物的水分运输、维持水平衡和逆境胁迫下胞内渗透的调控中起重要作用(于利刚等2011; Azad等2008; Carbrey和Agre 2009; Kammerloher等1994)。

harpin是由革兰氏阴性植物病原细菌产生的性质和功能相似的一类蛋白质, 是激发植物过敏反应(hypersensitive response, HR)的主要因子(Li等2011; Liu等2006)。研究发现, 外施harpin能诱导植物抗病、抗虫和耐旱, 促进植物的生长(Chen等2008a, b; Dong等2004, 2005; Zhang等2007)。进一步的实验证实, harpin蛋白的这些多效性是由于激活了多种不同的信号通路, 包括植物激素的转导、活性氧信号、离子通道、细胞程序性死亡、系统获得抗性或水杨酸和蛋白质激酶级联等(Dong等1999; Liu等2010; Lü等2011; Peng等2003, 2004; Sang等2012; Zhang等2011)。然而harpin蛋白从植物细胞外转运到细胞内的植物转位子是什么仍未阐释清楚。

实验室前期的研究结果发现, 拟南芥中的质膜内在蛋白AtPIP1;4能与水稻白叶枯病菌(*Xanthomonas oryzae* pv. *oryzae*)产生的harpin蛋白Hpa1互作(Li等2015)。进一步同源比对分析发现, 水稻(*Oryza sativa*)中与AtPIP1;4具有高度同源性的OsPIP1;3同样与Hpa1存在互作, 但是作用位点尚未研究清楚。本文采用片段缺失法以及改良后快速点突变方法构建缺失及点突变的OsPIP1;3蛋白载体, 采用膜酵母双杂交的方法研究突变载体与Hpa1的互作情况, 以确定OsPIP1;3蛋白与Hpa1的互作位点。

材料与方法

1 材料

Pfu DNA聚合酶、MutanBEST Kit、DNA marker以及去甲基化酶*Dpn*I购自TaKaRa公司, 琼脂糖购自Solarbio公司。模板pMetYCgate::*OsPIP1;3*为本实验室保存, 大肠杆菌(*Escherichia coli*)菌株DH5α和酿酒酵母(*Saccharomyces cerevisiae*)菌株NMY51 [*MATa his3delta200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3ura3::(lexAop)8-lacZ (lexAop)8-ADE2 GAL4*]均为本实验室保存。其他试剂均为分析纯。

2 实验方法

2.1 引物设计

应用引物设计软件Primer Premier 5, 以pMetYCgate::*OsPIP1;3*为模板, 设计含有不同缺失片段的载体引物(表1)以及点突变载体引物(表2)(王荣浩等2008)。

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表1 片段缺失载体引物序列及缺失区域

Table 1 The sequences of primers in deletion mutagenesis and their deletion regions

引物名称	引物序列(5′→3′)	缺失区域/aa
Δ1~94F	ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGTCGTTCGGCGGCATGATCTTCG	1~94
Δ1~94R	TCCGCCACCACCAACCACTTTGTACAAGAAAGCTGGGTAGTCCCGGCTCTTGAAGGGGATTG	
Δ94~144F	GCCATCTGCGGCGCCGGCGTGG	94~144
Δ94~144R	CCACGCGATGCCCTGGATCCCC	
Δ144~288F	ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGGAGGGGAAGGAGGAGGATGT	144~288
Δ144~288R	TCCGCCACCACCAACCACTTTGTACAAGAAAGCTGGGTACAGGCACTGCATCGCCATGTAG	
Δ156~178F	GGGCTCGGGGCGGAGATCGT	156~178
Δ156~178R	GAACCCCTTCACCACGCCGGC	
Δ208~288F	ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGGAGGGGAAGGAGGAGGATGT	208~288
Δ208~288R	TCCGCCACCACCAACCACTTTGTACAAGAAAGCTGGGTAGGAGTCCCTGGCGTTGCGCTTG	
Δ230~279F	AGAGCAATCCCCTTCAAGAGC	230~279
Δ230~279R	GGGGATGGTGGCGAGGTGGAC	
Δ246~288F	ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATGGAGGGGAAGGAGGAGGATGT	246~288
Δ246~288R	TCCGCCACCACCAACCACTTTGTACAAGAAAGCTGGGTAGGCGGCGCCGAGGCTGCG	

表2 快速点突变引物序列及其突变位点

Table 2 The sequences of primers in quick change mutagenesis and their mutation positions

引物名称	引物序列(5′→3′)	突变位点/aa
P255~257F	CCCACGCATGGCAACACCAGTGGATTTTCTGG	255, 256, 257
P255~257R	GAAAATCCACTGGTGTTGCCATGCGTGGGCGC	
P258~260F	CACGACCACAGGATGTTGTGGGTTGGTCCGTT	258, 259, 260
P258~260R	CCAACCCACAACATCCTGTGGTCGTGCCATGC	
P261~263F	GGATTTTCTGCATTCGTCCGTTCATCGGAGCG	261, 252, 263
P261~263R	GATGAACGGACGAATGCAGAAAATCCAGTGGT	
P264~266F	GGTTGGTGCGTACGTCGGAGCGGCACTGGCGG	264, 265, 266
P264~266R	GTGCCGCTCCGACGTACGCACCAACCCAGAAAAT	
P267~269F	CGTTCATCCGACCGACACTGGCGGCCATCTAC	267, 268, 269
P267~269R	GGCCGCCAGTGTCGGTCGGATGAACGGACCAAC	
P270~272F	CATCGGAGCGGCACAGGTGACCATCTACCACGT	270, 27, 272
P270~272R	GTAGATGGTCACCTGTGCCGCTCCGATGAACGG	
P273~275F	GATTGCTCTGATCACCACCACTTGGGAGGTGGCC	273, 274, 275
P273~275R	GCACTGGCGGCCACCTCCCAAGTGGTGGTGATCAG	
P276~279F	GGGGATTGCTCTGTTCTCCTCCTCGTGGTAGATGG	276, 277, 278, 279
P276~279R	CATCTACCACGAGGAGGAGAACAGAGCAATCCCC	

突变位点以下划线标记。

2.2 突变载体构建

片段缺失的载体构建: 以pMetYCgate::*OsPIP1;3*质粒为模板进行PCR扩增, 引物如表1所示; 方法参照MutanBEST Kit (code No. R401)说明书(http://www.takara.com.cn/DownLoad/R401.pdf)。

点突变载体的构建: pMetYCgate::*OsPIP1;3*质粒为模板, 应用突变位点的不完全匹配引物进行PCR扩增, 所用引物如表2所示。PCR反应体系为: *Pfu* DNA聚合酶1 μL、10×*Pfu*聚合酶缓冲液5 μL、2 mmol·L^{-1} dNTPs (4种脱氧核糖核苷三磷酸dATP、dCTP、dGTP和dTTP等浓度混合物) 2.5 μL、50 ng·μL^{-1}质粒模板1 μL、10 μmol·L^{-1}的正反向引物各1 μL, 补双蒸水至50 μL。PCR反应条件: 94°C 3 min, 1个循环; 94°C 1 min, 60°C 2 min, 68°C 15 min, 17个循环; 68°C 30 min, 1个循环。取所得的PCR产物10 μL用于琼脂糖凝胶电泳确定扩增产量, 然后在剩余的产物中加入3 μL *Dpn*I内切酶(该酶可以特异酶切完全甲基化的CATC序列), 以去除

模板质粒, 最后将*Dpn*I酶切后的质粒用于转化大肠杆菌DH5α, 涂板后于37°C倒置培养过夜(王荣浩等2008)。挑取阳性克隆培养, 参考萨姆布鲁克和拉塞尔(2005)的方法, 应用碱裂解法提取质粒DNA, 并用凝胶电泳检测质粒大小, 对大小正确的质粒进行测序分析。

2.3 膜酵母双杂交实验

split-ubiquitin膜蛋白酵母双杂交系统是将两个待检蛋白分别与突变且相互分离的泛素N端(即NubG, Nub的13位亮氨酸突变甘氨酸)和C端(即Cub)相融合, 两个待检测蛋白若存在互作, 可使被分裂的泛素N端和C端相互接近, 从而被泛素专一性蛋白酶(ubiquitin-specific proteases, UBPs)识别并导致报告蛋白(转录激活因子或酶等)解离释放, 进而判定蛋白互作情况。与传统的酵母双杂交系统相比, split-ubiquitin膜蛋白酵母双杂交系统不再依赖传统的核内转录激活机制, 可广泛用于细胞膜和细胞质内蛋白互作的研究。

本实验通过醋酸锂转化方法将OsPIP1;3-Cub+NubG-Hpa1、*m*OsPIP1;3-Cub+NubG-Hpa1 (片段缺失突变或点突变载体)、KAT1-Cub+NubG-KAT1 (阳性对照组)及KAT1-Cub+NubG-SUT2 (阴性对照组) 4个组合共转到NMY51酵母菌中, 将转化混合物分别涂布到SD-WL (二缺, 缺少色氨酸和亮氨酸的培养基)、SD-WLH (三缺, 缺少色氨酸、亮氨酸和组氨酸的培养基)及SD-WLAH (四缺, 缺少色氨酸、亮氨酸、组氨酸和腺嘌呤的培养基)平板上培养3~4 d后观察菌落生长情况, 在SD-WLH及SD-WLAH培养基上划线3次均能生长的克隆确定为阳性克隆。挑取数个单克隆菌落培养并进行X-Gal染色检测(萨姆布鲁克和拉塞尔2005)。

实验结果

1 基因片段缺失及定点突变载体的构建

以pMetYCgate::*OsPIP1;3*质粒为模板, 分别以表1和2所列序列为引物, 扩增pMetYCgate::*OsPIP1;3*质粒, 将PCR产物进行琼脂糖凝胶电泳验证。结果如图1所示, 片段大小正确, 条带可见, 测序正确, 成功获得了本实验所需要的含有突变位点的重组质粒。

2 互作区域的确定

首先, 应用膜酵母双杂交系统对OsPIP1;3三个缺失载体(pMetYCgate::*OsPIP1;3*$_{\Delta 1\sim 94}$、pMetYCgate::*OsPIP1;3*$_{\Delta 94\sim 144}$和pMetYCgate::*OsPIP1;3*$_{\Delta 144\sim 288}$)进行蛋白互作检测, 结果显示, Hpa1与OsPIP1;3的互作位点位于144~288 aa区域内(图2)。进一步地, 对144~288 aa范围内再次进行区段分割, 并用测序正确后的OsPIP1;3片段缺失突变体(pMetYCgate::*OsPIP1;3*$_{\Delta 156\sim 178}$、pMetYCgate::*OsPIP1;3*$_{\Delta 208\sim 288}$、pMetYCgate::*OsPIP1;3*$_{\Delta 230\sim 288}$、pMetYCgate::*OsPIP1;3*$_{\Delta 246\sim 288}$)进行酵母双杂交实验, 结果显示, Hpa1和OsPIP1;3的互作区域为246~279 aa, 该区段包含Loop E和TM6跨膜结构域(图2)。

3 OsPIP1;3点突变氨基酸序列分析

为确定具体的互作位点, 我们首先对TM6跨膜区段上的全部24个氨基酸进行了定点突变, 8个突变载体中每个包含3~4个突变氨基酸, 突变后的OsPIP1;3氨基酸排列如表3所示。

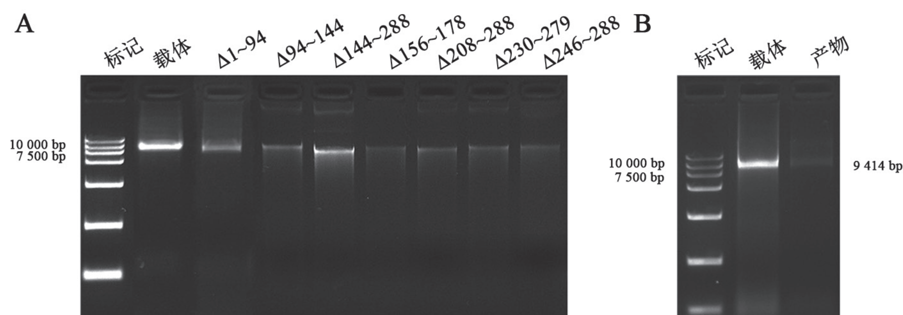

图1 含*OsPIP1;3*突变体质粒的电泳分析结果

Fig.1 Electrophoretic analysis of plasmids with and without an insert of *OsPIP1;3* mutants

A: 含有缺失片段的载体; B: 含有点突变的载体。标记: DL15000 marker; 载体: 含有OsPIP1;3序列的pMetYCgate质粒; Δ**: PCR扩增片段缺失产物; 产物: PCR扩增点突变产物。

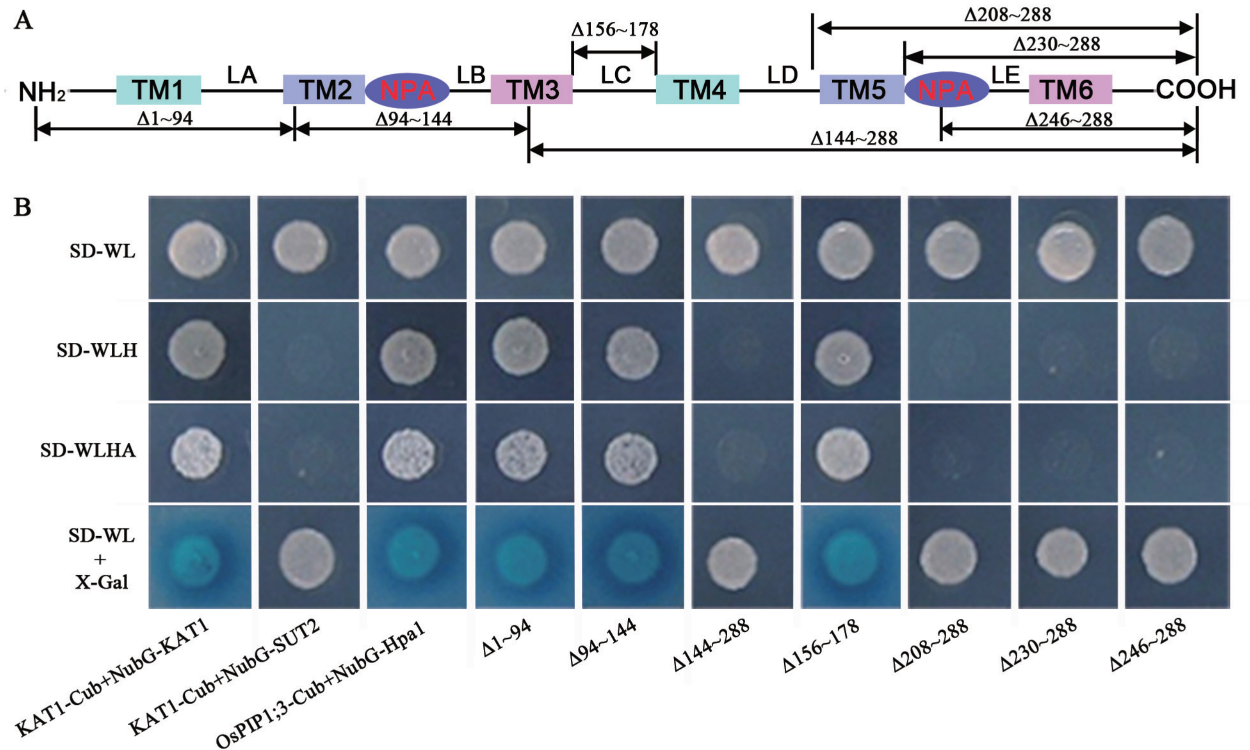

图2 Hpa1与OsPIP1;3互作区域的鉴定

Fig.2 Identification of Hpa1-interacting regions in the OsPIP1;3

A: 片段缺失载体位置示意图; B: 片段缺失载体酵母双杂交结果。

表3 Hpa1与OsPIP1;3突变载体的酵母双杂交分析

Table 3 Yeast two-hybrid tests of Hpa1 in combination with OsPIP1;3 or every mutant version of OsPIP1;3

互作组合	氨基酸序列	菌落生长			X-Gal染色
		二缺	三缺	四缺	
OsPIP1;3-Cub+NubG-Hpa1	HDHWIFWVGPFIGAALAAIYHVVVI	+	+	+	蓝色
$mu_{255\sim257}$OsPIP1;3-Cub+NubG-Hpa1	QHQWIFWVGPFIGAALAAIYHVVVI	+	+	+	蓝色
$mu_{258\sim260}$OsPIP1;3-Cub+NubG-Hpa1	HDHRMLWVGPFIGAALAAIYHVVVI	+	+	+	蓝色
$mu_{261\sim263}$OsPIP1;3-Cub+NubG-Hpa1	HDHWIFCIRPFIGAALAAIYHVVVI	+	+	+	蓝色
$mu_{264\sim266}$OsPIP1;3-Cub+NubG-Hpa1	HDHWIFWVGAYVGAALAAIYHVVVI	+	+	+	蓝色
$mu_{267\sim269}$OsPIP1;3-Cub+NubG-Hpa1	HDHWIFWVGPFIRPTLAAIYHVVVI	+	+	+	蓝色
$mu_{270\sim272}$OsPIP1;3-Cub+NubG-Hpa1	HDHWIFWVGPFIGAAQVTIYHVVVI	+	+	+	蓝色
$mu_{273\sim275}$OsPIP1;3-Cub+NubG-Hpa1	HDHWIFWVGPFIGAALAATSQVVVI	+	+	+	蓝色
$mu_{276\sim279}$OsPIP1;3-Cub+NubG-Hpa1	HDHWIFWVGPFIGAALAAIYHEEEN	+	−	−	白色

前缀“*mu*”代表OsPIP1;3突变体, 图4同; +表示有菌落, −表示无菌落, 突变后的氨基酸用下划线标记。

4 互作位点的筛选

实验中, 以含有点突变的OsPIP1;3为诱饵, 以Hpa1蛋白为猎物进行酵母双杂交检测。实验发现, 当276~279 aa由缬氨酸–缬氨酸–缬氨酸–异亮氨酸突变为谷氨酸–谷氨酸–谷氨酸–天冬酰胺后(图3), 含有该突变的OsPIP1;3 (*mu*OsPIP1;3)和Hpa1的酵母菌在三缺和四缺的培养基上不再生长且X-Gal处理不再显色, 而含有未经突变OsPIP1;3与Hpa1同时转酵母菌则能在三缺和四缺板上正常生长, 并且X-Gal处理显蓝色(图4)。上述结果表明, 276~279 aa很有可能就是OsPIP1;3和Hpa1蛋白的互作位点。

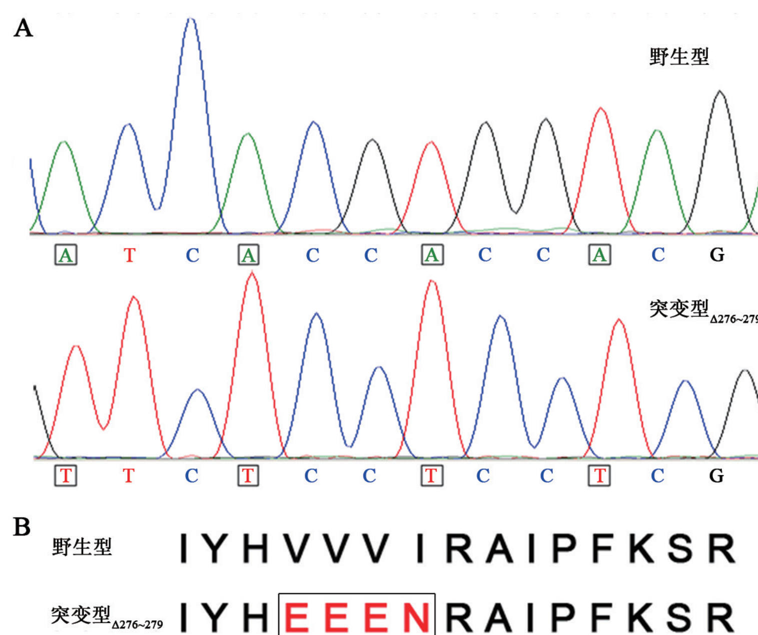

图3 突变质粒的DNA与氨基酸序列

Fig.3 DNA and amino acid sequences of the mutation plasmid

A: DNA测序序列; B: 突变后的氨基酸序列。

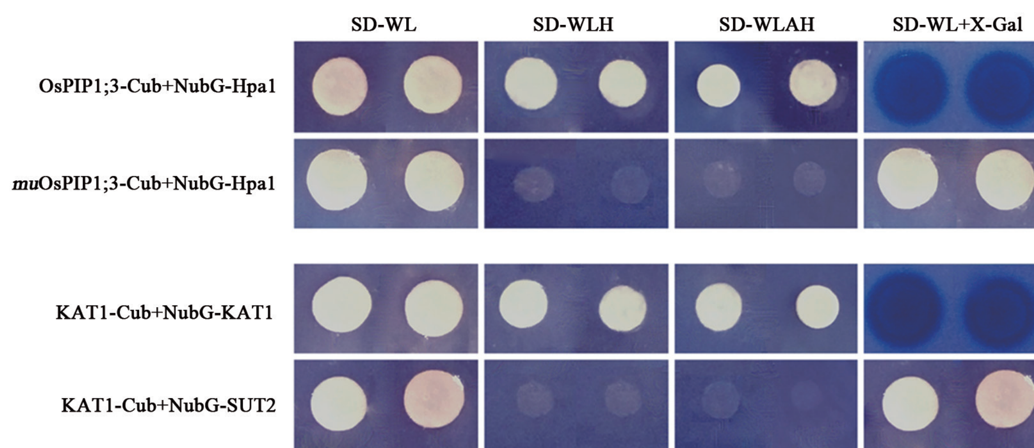

图4 Hpa1与OsPIP1;3突变载体的酵母双杂交实验及X-Gal染色结果

Fig.4 Yeast two-hybrid and X-Gal assays of Hpa1 and OsPIP1;3 or the mutant

OsPIP1;3-Cub+NubG-Hpa1为OsPIP1;3未突变; *mu*OsPIP1;3-Cub+NubG-Hpa1为OsPIP1;3的276~279 aa突变; KAT1-Cub+NubG-KAT1为正对照; KAT1-Cub+NubG-SUT2为负对照。

讨 论

之前的研究一直关注于PIPs如何完成水分子及溶质分子的运输及机理的阐述(Yaneff等2015; Yooyongwech等2013), 最近发现AQP能调控植物–病原物互作及植物防卫反应, 但机制还未得到研究(Tian等2016)。本文通过对OsPIP1;3进行片段缺失和点突变确定了互作区域以及一个可能的互作位点, 这为阐释OsPIP1;3与Hpa1的互作机制提供了基础。结果显示, 当loop E和TM6区段缺失后, OsPIP1;3$_{\Delta246\sim279}$不再与Hpa1互作; 而当同时突变TM6跨膜结构域中276~279位四个氨基酸后, OsPIP1;3与Hpa1的互作消失, 表明该位点氨基酸可能是互作位点(图5)。

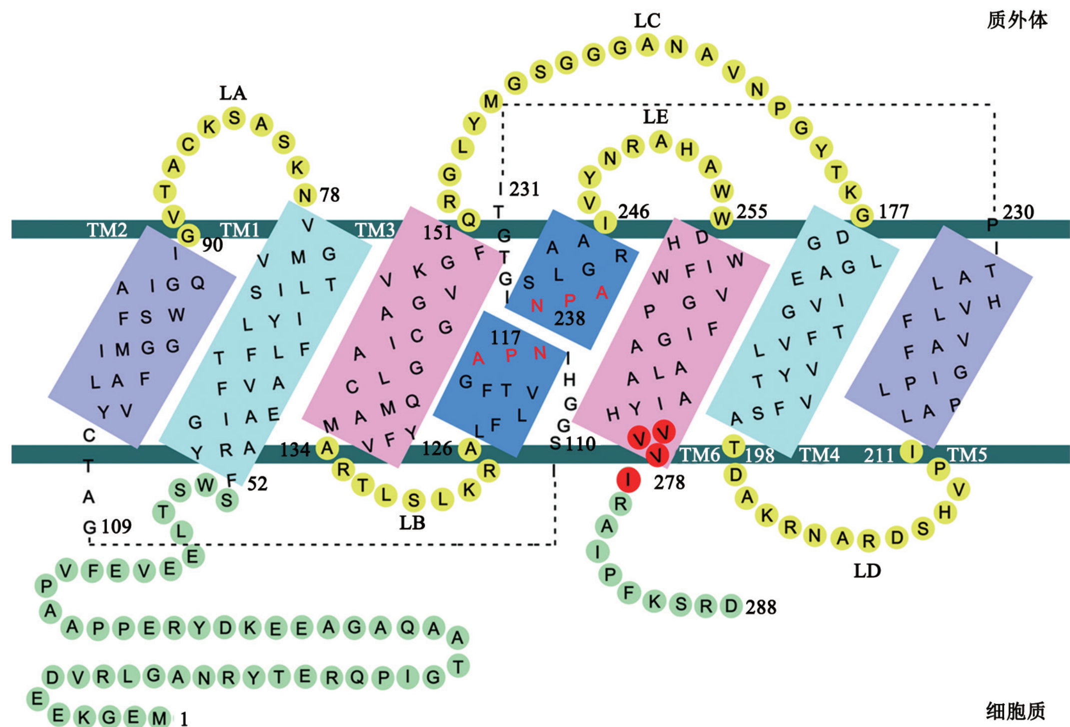

图5 OsPIP1;3拓扑结构模式图

Fig.5 A model of topological structure of OsPIP1;3

对于TM6跨膜结构域如何影响OsPIP1;3与Hap1蛋白的互作, 我们推测一种可能是276~279 aa的突变影响OsPIP1;3的三级拓扑结构, 酵母双杂交系统检测蛋白互作的灵敏性取决于诱饵与猎物蛋白的表达量, 可能酵母双杂交实验无法检测到变弱的OsPIP1;3与Hpa1的互作(曹炜2007; 郑立双等2013); 另一种可能是276~279 aa为OsPIP1;3与Hpa1的互作位点, Hap1很有可能是作为转位子蛋白, 而胞质膜上必然存在与转位子蛋白结合的特定蛋白(Bocsanczy等2008; Kvitko等2007; Li等2011), 而已有研究显示, 含有*harpin*的转基因植物也能表现出类似于低浓度喷洒harpin蛋白的生理效应, 表明harpin蛋白的作用位点很可能位于细胞内(Sang等2012; Choi等2013)。

为进一步确定176~179 aa的作用, 我们将进行包括双分子荧光互补实验在内的蛋白互作实验以确定实验结果的可靠性。OsPIP1;3与Hpa1蛋白互作位点的清晰阐释, 将为研究Hpa1如何诱导植物过敏反应的信号通路以及AQP是如何参与植物防卫反应的研究提供又一思路及理论参考。

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Studies on the interaction between aquaporin OsPIP1;3 of rice and harpin protein Hpa1 of *Xanthomonas oryzae* pv. *oryzae*

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Abstract: In this study, we generated mutant versions of rice (*Oryza sativa*) aquaporin OsPIP1;3 by fragment deletion and site-directed mutagenesis, respectively. When a split-ubiquitin–based yeast two-hybrid system was employed to analyze protein combinations of Hpa1 (a harpin protein of *Xanthomonas oryzae* pv. *oryzae*) with the canonical form and mutant versions of OsPIP1;3, interaction was found between Hpa1 and the canonical form, but not in the combination of Hpa1 with the mutant protein generated by deleting a short intracellular region called connecting loop E and transmembrane domain 6 (TM6). Analyses of additional substitutive protein mutants indicated that residues 276–279 in TM6 were also important for the molecular interaction. These data suggest that loop E and TM6 are determinants of OsPIP1;3 interaction with Hpa1 while particular residues 276–279 play crucial roles in the molecular interaction.
Key words: Hpa1; OsPIP1;3; site-directed mutagenesis; membrane-based yeast two-hybrid; protein interaction

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