拟南芥酵母双杂交文库构建及ADPG1互作蛋白筛选

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摘要 纤维素酶、半纤维素酶和果胶酶在植物果实发育和成熟过程中起着重要的作用。拟南芥开裂区多聚半乳糖醛酸酶1(ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE 1, ADPG1)是拟南芥角果开裂所必需的果胶酶, *ADPG1*在木质部的异位表达可以诱导拟南芥*ccr1*突变体病程相关(pathogenesis-related, *PR*)基因的表达。但ADPG1是如何促进*ccr1*释放激发子并诱导病害防御基因表达的尚不清楚。通过筛选其互作的蛋白, 可进一步探究ADPG1参与*ccr1*突变体激发子释放和诱导防御基因*PR1*表达的作用机制。该文以拟南芥野生型和突变体*ccr1*为材料, 通过提取总RNA, 分离mRNA, 合成双链cDNA, 依次构建酵母双杂交初级和次级文库, 构建诱饵载体pGBKT7-AtADPG1, 进行自激活检测, 与构建的次级文库共转化酵母感受态细胞, 筛选到1个与ADPG1相互作用的候选蛋白AtGRP5, 为进一步研究其参与植物诱导防御反应过程奠定了基础。

关键词 拟南芥; AtADPG1; 酵母文库构建; 酵母双杂交; 互作蛋白

Construction of Yeast Two-Hybrid Library of *Arabidopsis thaliana* and Screening of ADPG1 Interacting Proteins

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Abstract Cellulase, hemicellulase and pectinase play critical roles in the fruit development and ripening. ADPG1 (ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE 1) is an essential pectinase for silique dehiscence. In addition, the ectopic expression of *ADPG1* in xylem can induce the expression of *PR* (pathogenesis-related) gene in *Arabidopsis ccr1* mutant. However, the mechanism of how ADPG1 promotes the release of elicitors in *ccr1* mutant and induces the expression of defense-related gene is still unclear. To explore the role of ADPG1 involving in the release of elicitors and the induction of defense gene *PR1* in *ccr1* mutants, the interacting proteins with ADPG1 were screened. In this paper, the authors extracted the total RNA from wild-type and *ccr1 Arabidopsis*. Then, the extracted RNA was used to isolate mRNA and synthesize double-stranded cDNA. The primary and secondary libraries of yeast two-hybrid were constructed successively. The bait vector pGBKT7-AtADPG1 was constructed and tested for its self-activation. The pGBKT7- AtADPG1 was co-transformed into yeast competent cells with the secondary library, and a candidate protein named AtGRP5 that interacting with ADPG1 was identified. This study provided a basis for further study on the involvement of ADPG1 in plant defense response.

Keywords *Arabidopsis*; AtADPG1; construction of yeast library; yeast two-hybrid; interacting proteins

收稿日期: 2022-01-19 接受日期: 2022-03-07

国家自然科学基金(批准号: 32030010)资助的课题

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Received: January 19, 2022 Accepted: March 7, 2022

This work was supported by the National Natural Science Foundation of China (Grant No.32030010)

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多聚半乳糖醛酸酶(polygalacturonases, PGs)是一类广泛存在于植物界中的果胶水解酶, 因其具有降解果胶的特性, 在多种植物, 如烟草[1]、苜蓿[2]、芒果[3]、百合[4]、苹果[5-6]、番茄[7-9]、大豆[10]、辣椒[11]、草莓[12-13]中均有研究。PGs作用于果胶降解的最后一步, 切割果胶主链并释放低聚半乳糖醛酸(oligogalacturonides, OGs)[14-15]。根据PGs水解方式和作用底物的不同, 可将其分为三种类型, 内切多聚半乳糖醛酸酶(endo-PGs)、外切多聚半乳糖醛酸酶(exo-PGs)和鼠李糖多聚半乳糖醛酸酶(rhamno-PGs, RGs)[16-17]。PGs参与的果胶的降解不仅涉及到了植物器官的扩增、分离和开裂等过程, 还可以使细胞壁结构解体, 导致果实软化[18-19]。

开裂区多聚半乳糖醛酸酶1(ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE 1, ADPG1)是拟南芥中的一种果胶裂解酶, 归类为PGs糖苷水解酶家族28[20-21], 其水解类型为endo-PGs, 主要参与了角果和花药的开裂、脱落过程中的细胞壁修饰和果胶分解等过程, 其功能缺失突变会导致结实减少、果实不裂和花粉脱落减少[22]。*ADPG1*的表达集中于花药和成熟角果的裂开区(dehiscence zone, DZ)[23-24]。对*ADPG1*进行表达模式分析发现, *ADPG1*首先在角果受精的雌蕊中被检测到, 且其表达量随着角果发育的过程逐渐增加; 除此之外, 在开花前的花药中和种子脱落区也有表达[22]。在拟南芥中, 有1个和*ADPG1*高度同源的*ADPG2*基因。ADPG1和ADPG2已被证明是拟南芥角果开裂所必需的果胶酶[25], 两者在造成果荚破裂和种子脱落方面存在部分功能冗余[26]。

除了影响细胞的果胶降解外, *AtADPG1*还涉及到植物的防御反应。植物细胞壁是植物细胞抵抗外界病原体入侵的第一道防线, 其主要由纤维素、半纤维素、木质素和果胶等组成。对这些成分如木质素进行修饰, 会改变细胞壁的结构及完整性, 从而释放果胶衍生的损伤相关分子模式(damage-associated molecular pattern, DAMP)信号, 进而诱导植物防御基因的表达[27]。最新研究表明, *AtADPG1*在木质素含量降低的茎中参与潜在防御信号的释放[28]。在野生型拟南芥的茎中*ADPG1*并不表达, 但在木质素含量降低的HCT-RNAi和*ccr1*突变体的茎中, 检测到*ADPG1*的大量表达, 这在HCT-RNAi和*ccr1*突变体诱导*PR*基因表达的过程中起到了不可或缺的作用, 因此, 植物中*PR*基因的表达和细胞壁DAMP激发子的释放与*ADPG1*在木质部异位表达密切相关[29-31]。

*ADPG1*作为一个编码果胶裂解酶的基因在花药和角果开裂时以及改性木质素植物茎中表达, 这也突出了果胶和木质素在细胞壁完整性中的重要性, 研究ADPG1蛋白的互作蛋白及其作用机制, 有助于解释果胶和木质素在构成植物细胞壁防御网络中的作用。本研究通过构建ADPG1高表达的拟南芥酵母核文库, 利用酵母双杂交的方法筛选其互作蛋白, 为解析ADPG1参与细胞壁重塑并诱导防御反应过程奠定基础。

1 材料和方法

1.1 实验材料与试剂

拟南芥(*Arabidopsis thaliana*)野生型为Col-0(Columbia)生态型, 拟南芥突变体*ccr1*(SALK_123689); pGADT7和pGBKT7载体质粒来自本实验室; Clone-Miner™ II cDNA文库构建试剂盒购自Invitrogen公司; Oligotex mRNA Midi Kit购自QIAGEN公司; 大肠杆菌DH5α菌株、反转录试剂盒购自北京全式金生物技术有限公司; 限制性核酸内切酶购自NEB公司; 高保真Pfu-DNA聚合酶、多糖多酚植物基因组DNA提取试剂盒、质粒小提试剂盒、琼脂糖凝胶DNA回收试剂盒、单片段重组克隆试剂盒均购自天根生化科技(北京)有限公司; X-α-gal、酵母转化试剂盒购自上海欧易生物医学科技有限公司; 卡那霉素(Kan)、氨苄青霉素(Amp)、Trizol试剂、50× TAE试剂购自北京索莱宝科技有限公司; DL2000 DNA Marker购自北京艾德莱生物科技有限公司; 引物合成、DNA测序由北京擎科生物科技有限公司完成。

1.2 文库的构建

1.2.1 RNA的提取和mRNA的分离 根据*ADPG1*在拟南芥不同组织中的表达情况[28], 收集温室生长4周的野生型拟南芥开花前期、开花期的花及*ccr1*突变体的茎及开花期前后的花。用Trizol法提取混合材料的总RNA, 按照Oligotex mRNA Midi Kit说明书进行mRNA的分离和纯化, 从中取出10 mL左右, 用1%琼脂糖凝胶电泳检测, 进行质量评估, 以用于后续文库建立。

1.2.2 初级文库(Uncut型)的构建 参照Clone-Miner文库构建试剂盒的说明书, 将分离纯化后的mRNA进行反转录, 依次合成cDNA第一条链和第二

表1 本实验所用到的引物序列

Table 1 Primer sequences used in this experiment

引物名称 Primer name	引物序列(5′→3′) Primer sequence (5′→3′)
M13-F	GTA AAA CGA CGG CCA G
M13-R	CAG GAA ACA GCT ATG AC
pGADT7-DEST-F	TAA TAC GAC TCA CTA TAG GGC GAG CGC CGC CAT G
pGADT7-DEST-R	GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG ATT
ADPG1-F	CGG AAT TCT TGA GTA GCA ACG TTG ATG ATG GAT
ADPG1-R	CGG GAT CCC TTA AGA GCA TTT AGG AGA AAC GGT G

条链, 将cDNA与三框attB1重组接头连接后对cDNA进行分级分离及收集, 将收集后的cDNA和pDONR222载体进行BP重组反应, 产物通过电转化的方法转化大肠杆菌DH10B感受态细胞。取转化后细菌原液10 μL, 稀释100倍后, 从中取出50 μL, 再均匀涂布在LB平板上(含相应抗性Kan), 第2天对平板计数, 进行库容量鉴定。随机挑取平板上的24个克隆进行菌落PCR鉴定, 上游引物为M13-F, 下游引物为M13-R(表1), 反应产物用1%琼脂糖凝胶电泳检测。每毫升文库库容量(CFU/mL)=平板上的克隆数/50 μL×100倍×1×10^3(μL)。文库总库容量(CFU)=CFU(mL)×文库菌液总体积(mL); 重组率%=重组成功数量/克隆总数×100%。

1.2.3 次级文库的构建 检验合格的Uncut文库用肉汤培养基30 °C过夜振荡培养, 抽提质粒, 测*D*值并电泳检测, 质粒与pGADT7-DEST进行LR重组反应, 电转化大肠杆菌DH10B感受态细胞, 即为次级文库。对次级文库库容量、重组率和插入片段长度鉴定。菌落PCR鉴定时上游引物为pGADT7-DEST-F, 下游引物为pGADT7-DEST-R。从菌液中提取质粒用于后续酵母双杂交。

1.3 拟南芥ADPG1诱饵载体的构建、毒性和自激活检测

从TAIR网站上下载拟南芥*ADPG1*基因序列, 去掉*ADPG1*的N-端信号肽序列。以拟南芥突变体*ccr1*茎的cDNA为模板, 选择*Eco*R I和*Bam*H I这2个酶切位点, 设计同源重组特异性引物: 上游引物为ADPG1-F, 下游引物为ADPG1-R, 用高保真酶进行PCR反应, 扩增*ADPG1*序列, 回收PCR产物后, 与双酶切后的pGBKT7载体同源重组连接, 转化大肠杆菌, 筛选阳性克隆, DNA测序, 获得诱饵载体pGBKT7-AtADPG1。

挑取酵母Gold菌种划线于YPDA平板上, 按照步骤制备酵母Y2H感受态细胞, 以质粒pGBKT7-Lam/pGADT7-T为阴性对照、质粒pGBKT7-53/pGADT7-T为阳性对照、质粒pGBKT7为空载对照、pGBKT7-ADPG1/pGADT7为自激活检测组进行共转化, 四组均涂布于SD/-Leu/-Trp/X-α-gal(DDO/X)上, 自激活组另涂布在SD/-Leu/-Trp/-His/X-α-gal(TDO/X)和SD/-Leu/-Trp/-His/-Ade/X-α-gal/AbA(QDO/X/A)培养基上, 30 °C恒温培养箱中倒置培养3~4天, 观察结果。

1.4 酵母双杂交文库筛选

制备新鲜的酵母感受态细胞, 将10 μg pGADT7文库质粒与5 μg pGBKT7-AtADPG1共转化, 先涂布于SD/-Trp/-Leu/X-α-Gal/AbA固体培养基, 单克隆长至大小1~2 mm, 初筛完成, 挑选平板上的蓝色克隆再次转移到二次筛选培养基SD/-Trp/-Leu/-His/-Ade/X-α-Gal/AbA平板上。30 °C培养3~5天, 菌落生长至2~3 mm, 挑取阳性克隆, 并从中提取酵母质粒, 进行一对一验证。

2 结果与分析

2.1 总RNA的提取及mRNA的分离

采用Trizol法提取拟南芥野生型和*ccr1*突变体特定时期的混合样品RNA, 经琼脂糖凝胶电泳后, 28s和18s rRNA亮度和宽度约呈现2:1的比例, 条带清晰, 无拖尾现象, 说明RNA提取质量较好(图1A)。总RNA经分离和纯化后获得mRNA, 琼脂糖凝胶电泳检测结果显示分离的mRNA呈弥散性分布, 条带范围分布广, 长度分布在750~2 200 bp(图1B), 拖带最亮部分位于1 000~2 000 bp。表明分离纯化的mRNA质量良好, 利用纯化的mRNA获得双链cDNA, 进行分级分离并收集, 作为建库起始样品。

2.2 初级文库的构建及鉴定

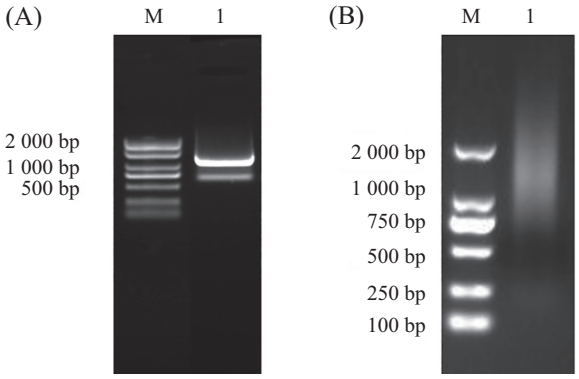
将cDNA与载体pDONR222连接重组后, 转入大肠杆菌, 将10 μL细菌原液稀释100倍后取50 μL涂布于LB平板(含Kan)上, 次日计数克隆总数约为1 500个(图2A), 根据公式计算得到初级文库库容量约为1.20×10^7 CFU。随机挑取24个克隆进行菌落PCR鉴定, 琼脂糖凝胶电泳检测结果(图2B)显示, 存在1个空载, 重组率为96%, 片段之间大小差异明显, 分布在1 000~2 000 bp, 平均长度大于1 000 bp, 说明文库插入片段重组率高, 质量较好, 达到初级文库要求, 可用于后续次级文库的构建。

2.3 次级文库的构建及鉴定

将经验证合格的初级文库菌液30 °C过夜培养, 进行质粒抽提, 与pGADT7-DEST载体进行LR反应, 重组产物电转化大肠杆菌DH10B, 得到次级文库菌液。将10 μL细菌原液稀释100倍后取50 μL, 涂布于LB平板(含Kan)上, 次日计数克隆总数约为1 300个(图3A), 根据公式计算得到次级文库库容量约为1.04×10^7 CFU。随机挑取24个克隆进行PCR检测, 琼脂糖凝胶电泳检测结果(图3B)显示, 重组率为100%, 片段大小不一, 存在明显差异, 说明文库的多态性较好, 条带基本都大于750 bp, 主要分布在1 000~2 200 bp, 说明其长片段占比较大, 序列完整性良好。综上所述, 检测结果表明该文库完整性好、质量高, 可用于后续文库筛选等试验。

2.4 诱饵载体构建与自激活检测

将切掉信号肽的*ADPG1*基因扩增后与双酶切后的pGBKT7用同源重组法连接, 转化大肠杆菌, 挑阳性克隆测序后, 获得正确质粒。以质粒pGBKT7-Lam/pGADT7-T、pGBKT7-53/pGADT7-T、pGBKT7、pGBKT7-ADPG1/pGADT7分别为阴性对照、阳性对照、空载对照、自激活检测组共转化到

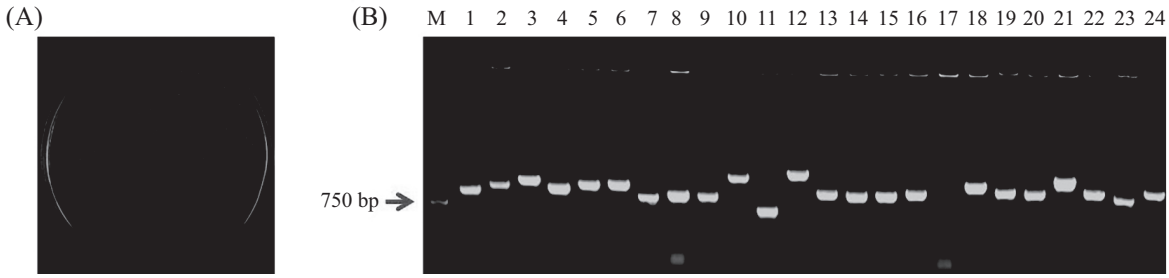


A: 拟南芥总RNA琼脂糖凝胶电泳, M: DL2000 DNA Marker; 1: 总RNA样品; B: 分离后mRNA琼脂糖凝胶电泳, M: DL2000 DNA Marker; 1: 分离后的mRNA样品。

A: agarose gel electrophoresis of *Arabidopsis* total RNA, M: DL2000 DNA Marker; 1: total RNA sample; B: agarose gel electrophoresis of isolated mRNA, M: DL2000 DNA Marker; 1: the mRNA sample after separation.

图1 总RNA的提取及mRNA的分离

Fig.1 Extraction of total RNA and isolation of mRNA



A: 初级文库库容量鉴定; B: 初级文库重组率鉴定; M: DL2000 DNA Marker; 1~24: 1~24号初级文库克隆菌落PCR扩增产物。

A: capacity determination of primary library; B: identification of primary library recombination rate; M: DL2000 DNA Marker; 1-24: clone colony PCR amplification products of primary library 1-24.

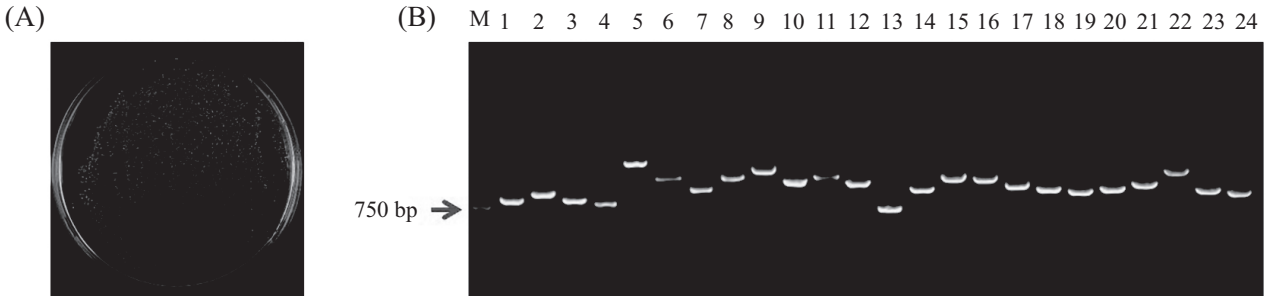
图2 初级文库的构建及鉴定

Fig.2 Construction and analysis of primary library

Y2H酵母感受态细胞中, 四组均涂布于SD/-Leu/-Trp/X-α-gal(DDO/X)上, 自激活组另涂布在SD/-Leu/-Trp/-His/X-α-gal(TDO/X)和SD/-Leu/-Trp/-His/-Ade/X-α-gal/AbA(QDO/X/A)培养基上, 30 ℃培养3~4天后, DDO/X板上四组均有生长, 其中只有阳性对照菌落呈现蓝色, 其余为白色菌落(图4A~图4D), 说明阳性对照、阴性对照, 空载对照实验成功, pGBKT7-AtADPG1成功转入酵母菌株; TDO/X和QDO/X/A板上自激活组均无生长(图4E和图4F), 说明该诱饵蛋白在Y2H酵母菌株中无自激活活性, 可进行后续筛选试验。

2.5 ADPG1互作蛋白筛选及验证

将pGBKT7-ADPG1质粒和构建的酵母双杂交文库质粒共转Y2H酵母感受态细胞, 转化后在SD/-Leu/-Trp/-His/X-α-Gal平板上稀释100倍培养3~5

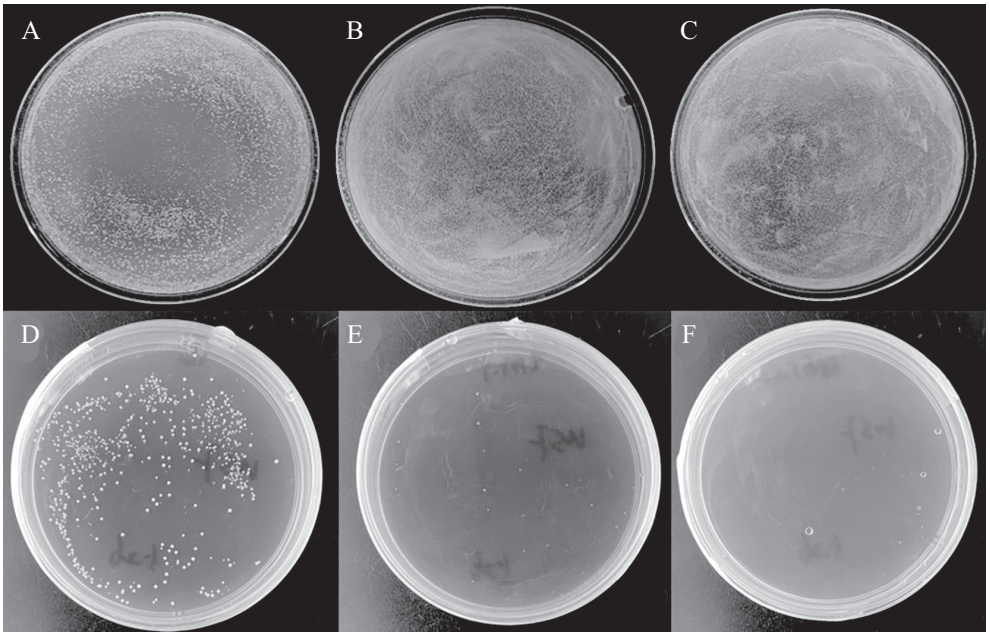


A: 次级文库库容量鉴定; B: 次级文库重组率鉴定; M: DL2000 DNA Marker; 1~24: 1~24号次级文库克隆菌落PCR扩增产物。

A: capacity determination of secondary library; B: identification of secondary library recombination rate; M: DL2000 DNA Marker; 1-24: clone colony PCR amplification products of secondary library 1-24.

图3 次级文库的构建及鉴定

Fig.3 Construction and analysis of secondary library

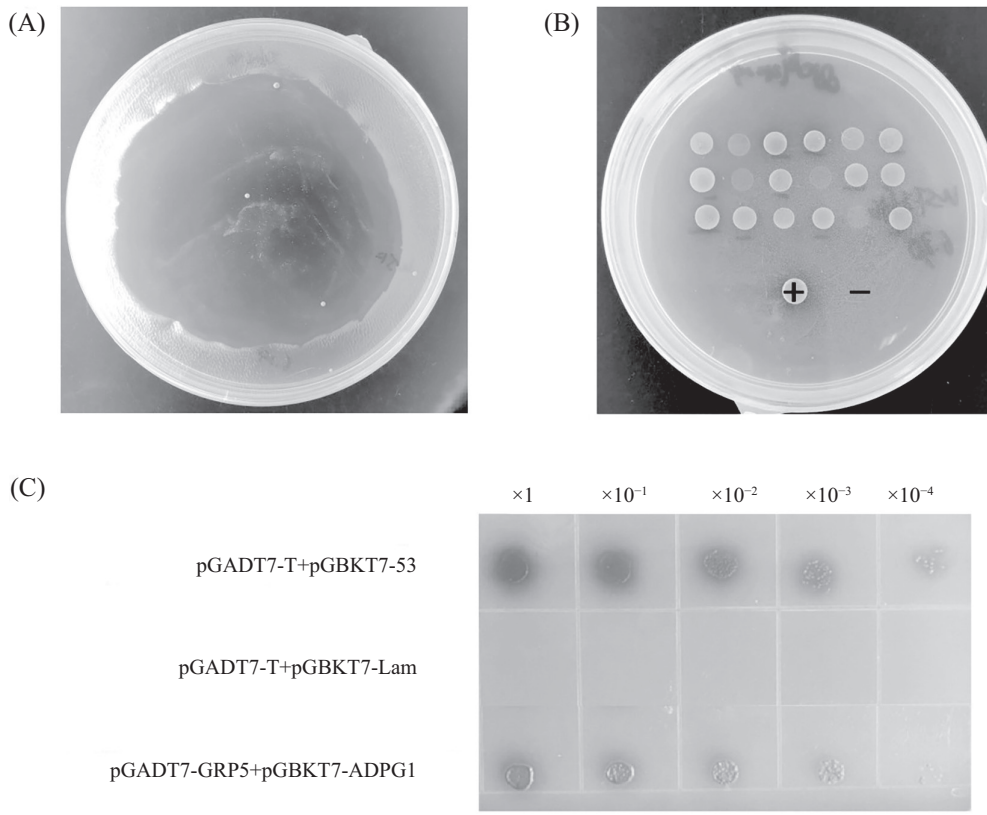


A: 阳性对照, pGBKT7-53/pGADT7-T, 涂布在DDO/X上; B: 阴性对照, pGBKT7-Lam/pGADT7-T, 涂布在DDO/X上; C: 空载对照, pGBKT7, 涂布在DDO/X上; D: 自激活实验组, pGBKT7-ADPG1/pGADT7, 涂布在DDO/X上; E: 自激活实验组, pGBKT7-ADPG1/pGADT7, 涂布在TDO/X上; F: 自激活实验组, pGBKT7-ADPG1/pGADT7, 涂布在QDO/X上。

A: positive control, pGBKT7-53/pGADT7-T, spreads plate of DDO/X; B: negative control, pGBKT7-Lam/pGADT7-T, spreads plate of DDO/X; C: empty vector, pGBKT7, spreads plate of DDO/X; D: self-activation test group, pGBKT7-ADPG1/pGADT7, spreads plate of DDO/X; E: self-activation test group, pGBKT7-ADPG1/pGADT7, spreads plate of TDO/X; F: self-activation test group, pGBKT7-ADPG1/pGADT7, spreads plate of QDO/X.

图4 诱饵载体自激活检测

Fig.4 Self-activation test of the bait vector



A: 初次筛选; B: 二次筛选,“+”表示阳性克隆, “–”表示阴性克隆; C: 候选蛋白一对一验证。

A: initial screening; B: secondary screening, “+” indicates positive clones,“–” indicates negative clones; C: one to one validation of candidate proteins.

图5 AtADPG1互作蛋白的筛选与验证

Fig.5 Screening and verification of AtADPG1 interacting proteins

表2 *AtGRP5*的简介

Table 2 Introduction to *AtGRP5*

基因号 Gene ID	蛋白号 Protein ID	蛋白名称 Protein name	功能 Function
AT3G20470.1	NP_188682.1	AtGRP5 (glycine-rich protein 5)	Positive regulation of cell growth; positive regulation of organ growth; response to abscisic acid; response to flooding

天, 初筛共获得18个蓝色的酵母克隆(图5A)。挑取初筛平板上的蓝色阳性克隆转移到SD/-Leu/-Trp/-His/-Ade/X-α-gal/AbA平板上, 其中有10个蓝色克隆(图5B)。对10个阳性克隆进行测序, 测序成功后, 将10个pGADT7-互作蛋白与pGBKT7-ADPG1进行一对一验证, 转化酵母后在四缺板上培养, 得到1个阳性克隆, 对其进行梯度稀释(图5C)。

2.6 ADPG1互作的候选蛋白功能分析

对阳性克隆进行基因测序分析, 在TAIR网站进行BLAST, 比对序列得到1个基因, 名为*AtGRP5*(表2)。AtGRP5定位于液泡, 是一种富含甘氨酸的蛋白质, 过表达*AtGRP5*的拟南芥花序轴和根均有伸长的表型, 抑制表达则表型相反, 这表明AtGRP5可能通过促进细胞伸长参与器官生长过程[32]。

3 讨论与结论

本研究通过收集拟南芥野生型和*ccr1*突变体中*ADPG1*特异表达的组织材料, 提取了总RNA, 构建了质量合格的酵母核文库。构建诱饵蛋白pGBKT7-AtADPG1并对其进行毒性和自激活检测, 将酵母文库质粒与诱饵蛋白质粒共转化酵母感受态细胞, 对初筛到的蛋白进行一对一验证, 筛选到1个可能与AtADPG1互相作用的候选蛋白AtGRP5。

植物富含甘氨酸蛋白质(glycine-rich protein, GRP)

是广泛存在于植物细胞壁上的一种重要结构蛋白, GRPs主要位于植物的维管组织中[33]。*GRP*基因的表达具有组织特异性并受发育阶段特异性及多种环境因素的调控[34]。*GRP*参与植物发育过程、生物和非生物胁迫的反应。水稻*OsGRP3*通过调节干旱胁迫下ROS相关基因的mRNA稳定性来减少ROS积累, 从而赋予水稻干旱耐受性[35]。过表达拟南芥*AtGRP7*可诱导与水杨酸(salicylic acid, SA)介导的免疫相关的*PR1*、*PR2*和*PR5*表达, 并增强拟南芥对*Pto*(*Pseudomonas syringae* pv. *tomato*) DC 3000的抗性[36]。

本研究筛选到的ADPG1候选互作蛋白基因*AtGRP5*, 编码一种富含甘氨酸的蛋白质, 在未成熟的种子荚中表达丰富, 茎和叶中次之, 在根或花中未检测到表达, 其响应脱落酸和水杨酸, 能促进细胞伸长, 参与调控植物生长和防御反应[37-39]。研究表明, *AtGRP3*和细胞质膜定位激酶相关蛋白磷酸酶(kinase-associated protein phosphatase, KAPP)通过与拟南芥细胞壁相关蛋白激酶1(cell wall-associated kinase 1, WAK1)互作在OG触发的防御基因表达和生产中的起负调控作用[40]。*AtGRP5*和*AtGRP3*同属拟南芥GRP家族, 我们推测, *ADPG1*与*GRP5*相互作用可能促进了WAK家族蛋白与OG的结合, 诱导了*PR1*的表达, 抑制了细胞的伸长, 从而导致了*ccr1*突变体植株矮小的表型。

本文所用的酵母双杂交技术(yeast two-hybrid, Y2H)是近年来研究蛋白之间相互作用的主要生物技术之一, 该方法高效快捷、成本低, 多用于研究微生物、植物和动物中的互作蛋白[41]。但该技术存在一定的局限性, Y2H验证的互作蛋白定位于细胞核内, 不适用于验证依赖翻译后加工的蛋白; Y2H还存在假阳性的问题, 主要是由所研究蛋白的特性而引起的, 如有些蛋白本身具有激活转录的功能, 有些可以与其他蛋白形成稳定的复合物, 从而激活转录[42]。由于Y2H结果存在假阳性的风险, ADPG1与GRP5的蛋白互作还需进一步验证, 后续可以利用更多的蛋白互作技术如双分子荧光互补实验(bimolecular fluorescence complementation, BIFC)和免疫共沉淀实验(co-immunoprecipitation, Co-IP)检测互作, GRP5是否通过与WAK形成复合体从而与ADPG1产生互作也需要进一步探索。综上所述, 本研究的结果为进一步探寻拟南芥基因*ADPG1*的分子作用机制和生物学功能提供了线索, 为后续研究提供了基础。

——致谢

感谢Richard A. DIXON教授对本论文选题和思路上的指导, 感谢于可济博士和宋玉双博士在论文写作和修改中提供的宝贵意见!

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