异源表达N-端融合eYFP标签的GAAPs增强拟南芥对ER胁迫敏感性

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摘要: 高尔基体抗凋亡蛋白GAAPs (Golgi anti-apoptotic proteins)在真核生物中高度保守, 是动物细胞中抑制胁迫诱导凋亡的BAX inhibitor-1类因子。反向遗传学研究表明, 拟南芥GAAPs也能够抑制ER胁迫诱导的细胞死亡。为进一步在蛋白质水平研究GAAPs的功能和作用机制, 通常使用基因融合标签的表达转化植株, 但要考虑所使用的标签是否影响蛋白的功能。为此, 我们分别构建了GAAP1和GAAP4的N末端融合eYFP、无eYFP标签的拟南芥过量表达转化子。通过检测eYFP-GAAP1/GAAP4以及*gaap1*突变体对ER胁迫敏感性, 发现eYFP-GAAP1/GAAP4过量表达与*gaap1*突变体类似, ER胁迫下的存活率降低, 细胞死亡增强。定量PCR分析表明eYFP-GAAP1过量表达抑制急性或长期ER胁迫下未折叠蛋白响应(unfolded protein response, UPR)通路相关基因的表达。这些结果表明GAAPs的N-端融合eYFP标签影响其在ER胁迫抗性以及抗细胞死亡中的功能。

关键词: 拟南芥; GAAPs; ER胁迫; 细胞死亡; 未折叠蛋白响应

植物会遭受各种环境胁迫, 并进化出多种生存机制应对胁迫(Asensi-Fabado等2017)。在严重或持续的胁迫下, 当植物的适应性反应无法修复细胞损伤时, 植物整体或局部将引发程序性细胞死亡(programmed cell death, PCD)。细胞的适应机制和生存机制是维持细胞内稳态的必要条件。内质网(endoplasmic reticulum, ER)是蛋白质加工成熟和脂质合成的主要细胞器之一, 环境胁迫会导致未折叠或错误折叠蛋白积累在内质网中, 造成内质网胁迫(ER stress), 定位于ER上的受体蛋白能够感知ER胁迫并激活未折叠蛋白响应(unfolded protein response, UPR)以缓解胁迫。哺乳动物细胞具有ER跨膜受体起始的3条UPR信号通路, 即IRE1 (inositol-requiring enzyme 1)、PERK (protein kinase RNA-activated-like ER kinase)和ATF6 (activating transcription factor 6), 通过维持体内平衡以促进细胞存活。IRE1能够剪接bZIP类转录因子XBP1 (X-box binding protein 1) mRNA, 从而上调促进蛋白折叠相关基因的表达; 也能够降解内质网中的mRNA, 即依赖IRE1调控的降解途径(regulated IRE1-dependent decay, RIDD); 还能调控细胞自噬降解并循环利用受损的内质网(Tirasophon等1998; Yoshida等2001; Calfon等2002; Yorimitsu和Klionsky 2007; Hollien等2009; Tam等2014)。ER胁迫下, ATF6转运至高尔基体, 经位点1和位点2的蛋白酶(S1P和S2P)加工处理, 最终定位于细胞核以激活蛋白质折叠相关基因的表达(Haze等1999; Yoshida等2000)。PERK通过调控翻译起始因子2a以及削弱翻译过程, 从而降低内质网的蛋白负荷(Harding等2000; Walter和Ron 2011)。在植物中已发现其中的两条信号通路, 分别由受体IRE1及其下游bZIP60 (XBP1同源物)和bZIP28 (ATF6同源物)介导(Iwata等2008; Nagashima等2011; Deng等2011, 2013; Iwata和Koizumi 2012)。

当内环境稳态无法被修复时, ER胁迫受体也会诱导PCD的发生(Walter和Ron 2011; Mishiba等2013; Ruberti等2018)。在哺乳动物细胞中, IRE1与不同的因子结合表现出不同的功能, 包括Bcl-2家族成员和BI-1 (BAX inhibitor-1) (Hetz和Glimcher 2009; Lisbona等2009; Woehlbier和Hetz 2011; Hetz 2012)。Bcl-2家族中的促凋亡和促生存蛋白是哺乳动物细胞中决定生死存亡的关键因子, 而在植物中鲜少发现抵抗PCD的Bcl-2家族蛋白。BI-1在真核生物中高度保守, 与Bcl-2因子互作从而抑制细胞凋亡。植物中存在动物BI-1同源蛋白, 在各种胁迫下表现出相似的细胞死亡抗性(Watanabe和Lam 2006, 2008)。BI-1具有6种同源蛋白亚家族, 命名为TMBIM (transmembrane BAX inhibitor motif containing) 1~6和1b, 根据系统发育分析, TMBIM

收稿 2018-04-09 **修定** 2018-05-14
资助 国家自然科学基金(31670271)和上海市自然科学基金(15ZR-1410900)。
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中5个成员合称为LFG (Lifeguard)家族(Hu等2009; Carrara等2012, 2017)。TMBIM4 (LFG4)也称为高尔基抗凋亡蛋白(Golgi anti-apoptotic proteins, GAAPs), 在真核生物中高度保守存在(Gubser等2007; Carrara等2017)。拟南芥中的5个GAAPs亚家族成员在一些报道中也称为AtLFG1~5。植物GAAPs在非生物胁迫诱导的PCD调控的作用尚未明确。我们在近期研究中发现, GAAP1/LFG1和GAAP3参与抵抗ER胁迫诱导的细胞死亡(Guo等2018)。为进一步研究GAAPs的功能和作用机制, 我们构建了GAAPs融合eYFP标签的表达载体, 而标签是否影响基因功能尚不清楚。本文通过整体和细胞水平分析GAAP1或GAAP4的N端融合eYFP的ER胁迫敏感性, 通过转录水平分析过量表达GAAP1 N-端融合eYFP对UPR信号通路的影响。

1 材料与方法

1.1 植物材料与生长条件

拟南芥[*Arabidopsis thaliana* (L.) Heynh.]野生型Col (Columbia-0)、突变体*gaap1-1*和*gaap1-2*详见Guo等(2018)文献。种子于4°C春化2~3 d, 在1/2MS培养基(1%蔗糖, 0.6%琼脂)中培养, 培养条件是16 h光照射, (23±2)°C。

为了研究幼苗的ER胁迫敏感性, 将消毒后的种子于含有低浓度衣霉素(tunicamycin, TM)的培养基中, 或者将在滤纸上正常生长3 d的幼苗转移至含有不同浓度的TM或者DTT (dithiothreitol)的培养基中培养不同时间, 测定幼苗的鲜重或根长, 并计算抑制率, 所有数据都来自至少3次独立的重复实验。

1.2 质粒构建与转化植株鉴定

将*GAAP1*基因的开放阅读框导入载体pMon-530 (Monsanto, USA), 构建35S启动子*GAAP1*过量表达质粒。GAAP1和GAAP4的N端融合eYFP标签并分别与pMon530载体相连, 获得eYFP-GAAP1和eYFP-GAAP4。拟南芥转化子获得参考Guo等(2018)文献。

1.3 组织化学和显微镜观察

细胞死亡通过台盼蓝染色检测(Duan等2010)。使用3,3′-二氨基联苯胺(3,3′-diaminobenzidine, DAB)染色检测组织中依赖过氧化物酶产生的内源性H_2O_2含量(Thordal-Christensen等1997)。每种染色分析都有4~5次独立的重复实验, 每次镜检每种材料至少20个样本。

1.4 实时荧光定量PCR (qPCR)

材料的处理方法: 将正常生长7 d的幼苗浸泡于含有不同浓度TM的1/2MS液体培养基, 对照组培养基中含有0.1% DMSO。液氮冷冻组织并用TRIZOL (Takara)提取总RNA, 采用反转录试剂盒(Toyobo, Japan)获得cDNA。qPCR分析的UPR基因的相对表达量数据来自于3~6次独立的重复实验(Schmittgen和Livak 2008; Li等2013)。基因引物见Guo等(2018)文献。通过Tukey-hornest双向差异分析, 确定不同基因型植株之间的显著性差异。

2 实验结果

2.1 过量表达N-端融合eYFP标签的GAAP1增强植物对ER胁迫的敏感性

我们已经发现GAAP1和GAAP3在植物抵抗ER胁迫中存在功能冗余, 在TM或DTT诱导的ER胁迫中, GAAP1和GAAP3突变体对ER胁迫的敏感性增强, 而过量表达2个基因则降低植株的死亡率、抑制细胞死亡(Guo等2018)。为了进一步研究GAAPs在植物中的功能及机制, 我们获得GAAP1的N-端融合eYFP的过量表达转化子(eYFP-GAAP1) (图1), 在正常条件下, GAAP1的过量表达株系的生长都没有明显表型。分析比较eYFP-GAAP1、*gaap1-1*和*gaap1-2*的ER胁迫敏感性, 生长于含有0.08 μg·mL^{-1} TM的培养基中的绿色健康幼苗比例降低, 与之前的研究结果一致的是*gaap1-1*和*gaap1-2*突变体的健康幼苗比率显著低于Col (Guo等2018), 而与野生型Col相比, eYFP-GAAP1株系在TM处理下表现出更加严重的生长缺陷(图2)。

为了排除TM对于种子萌发的影响, 将正常萌发3 d的幼苗转移至含有TM的培养基(0.3~0.5 μg·mL^{-1})中生长10 d, 分析死亡率和平均鲜重抑制率发现, eYFP-GAAP1幼苗比*gaap1*突变体的生长抑制更加严重(图3-A~C)。测量在0.1 μg·mL^{-1} TM处理下幼苗的主根根长, 结果发现与*gaap1gaap3*相似, eYFP-GAAP1在TM处理下的根长抑制率增加, 而GAAP1过量表达株系的根长抑制率减少(图3-D)。这些数据表明GAAP1提高ER胁迫抗性, 而eYFP

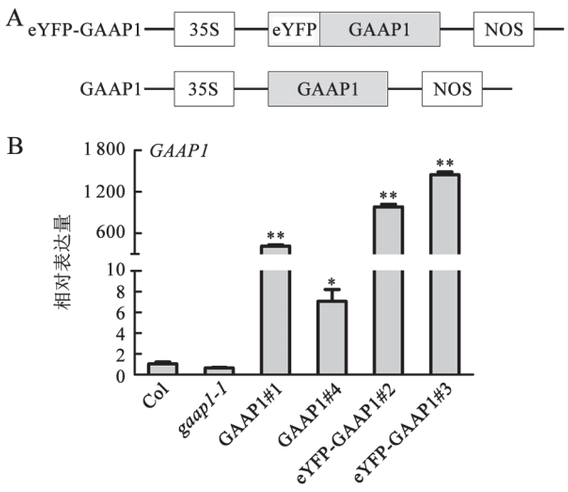

图1 *GAAP1*在突变体及转基因株系中的表达水平

Fig.1 *GAAP1* transcripts level in mutants and transgenic lines

A: 过量表达载体构建信息; B: qRT-PCR检测生长1周的材料中*GAAP1*的表达水平(*t*检验, *: P<0.05, **: P<0.01)。

融合GAAP1过量表达则提高了植株的ER胁迫敏感性。

2.2 N-端融合eYFP标签的GAAP1异源表达增强ER胁迫诱导的细胞死亡

为了进一步明确eYFP融合GAAP1对于细胞死亡的影响, 我们检测了*gaap1gaap3*和eYFP-GAAP1对于TM的响应。将竖直培养3 d的Col、*gaap1gaap3*和eYFP-GAAP1幼苗转移至含0和0.03 μg·mL^{-1} TM的培养基上处理4 d, 采用DAB染色检测子叶细胞活性氧水平、台盼蓝染色检测细胞死亡。与Col相比, 在eYFP-GAAP1与*gaap1gaap3*双突变体细胞中检测到更强的染色信号(图4), *gaap1gaap3*双突变体细胞死亡和活性氧增强的结果与之前研究结果一致(Guo等2018), 这表明eYFP融合GAAP1的异源表达促进ER胁迫下的细胞死亡。

2.3 N-端融合eYFP的GAAP4过量表达增强植株的ER胁迫敏感性

为了检测eYFP融合其他GAAPs同源基因是否影响植株的ER胁迫敏感性, 我们构建了eYFP-GAAP4过量表达转化子。在DTT或TM诱导的ER胁迫下, 相比Col, eYFP-GAAP4过量表达转化子植株的死亡率和细胞膜损伤程度都增加(图5), 这表明eYFP位于GAAP4的N端也增强植株的ER胁迫敏感性。

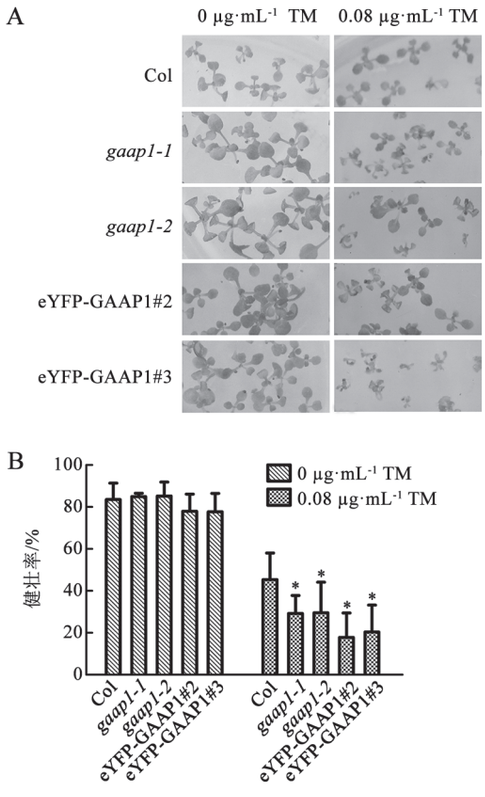

图2 GAAP1突变与eYFP-GAAP1过量表达增强植株对TM的敏感性

Fig.2 Mutation of GAAP1 and overexpressing eYFP-tagged GAAP1 enhanced plants sensitivity toward TM

A: 不同材料在含TM的培养基中生长10 d的表型; B: 绿色植株比例(χ^2检验, *: P<0.05, n=150)。

2.4 eYFP融合GAAP1的异源表达削弱ER胁迫下UPR相关基因的表达

eYFP-GAAP1能够增强植株对ER胁迫的敏感性, 我们进一步检测了eYFP-GAAP1蛋白是否影响UPR通路。首先, 我们采用高浓度TM (5 μg·mL^{-1})处理4 h的急性ER胁迫, 通过实时定量PCR检测根系中UPR激活的标记基因表达水平。除了eYFP-GAAP1和Col的根系中*AtPDI*的基因表达都有所上调外, 其他常见UPR激活标记基因*AtBIP3*、spliced *AtbZIP60* (*bZIP60s*)、unspliced *AtbZIP60* (*bZIP60u*)、*AtCNX1*和*AtCRT* (Martinez和Chrispeels 2003; Williams等2010)在eYFP-GAAP1幼苗中的表达上调都受到抑制(图6)。

为进一步分析持续ER胁迫是否影响UPR的变化模式, 我们采用0.5 μg·mL^{-1} TM处理4~36 h, 检测

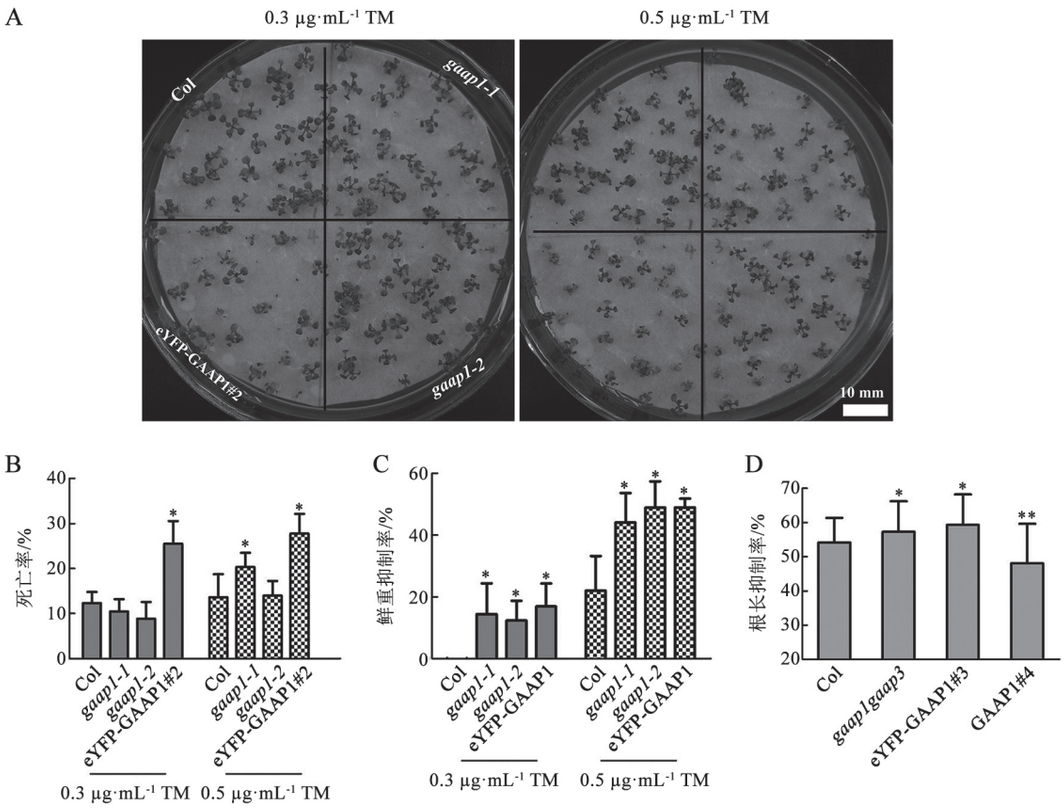

图3 GAAP1突变与eYFP-GAAP1过量表达增强幼苗对ER胁迫的敏感性

Fig.3 Mutation of GAAP1 and overexpressing eYFP-tagged GAAP1 increased the sensitivity of seedlings to ER stress

A~C: 生长3 d的幼苗转移至含TM的培养基生长10 d的表型(A)、植株死亡率(B)和平均鲜重抑制率(C) (*: P<0.05, n≥160)。D: 在含0.1 μg·mL^{-1} TM的培养基生长8 d的主根长抑制率(*: P<0.05, **: P<0.01, n>80)。

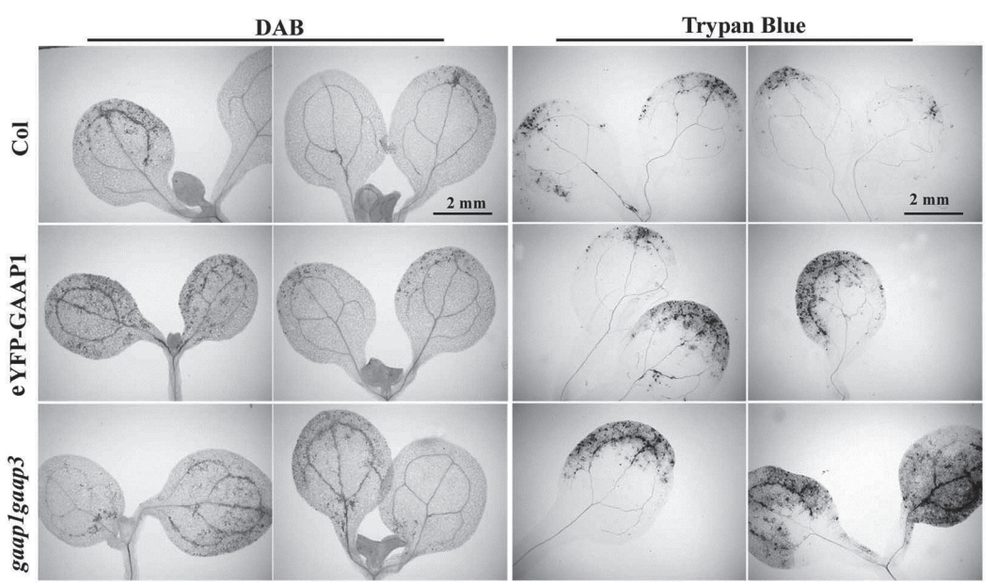

图4 GAAP1和GAAP3突变以及eYFP-GAAP1的异源表达促进ER胁迫诱导的细胞死亡

Fig.4 Mutations in GAAP1 and GAAP3, and ectopic expressing eYFP-tagged GAAP1 enhanced cell death induced by ER stress

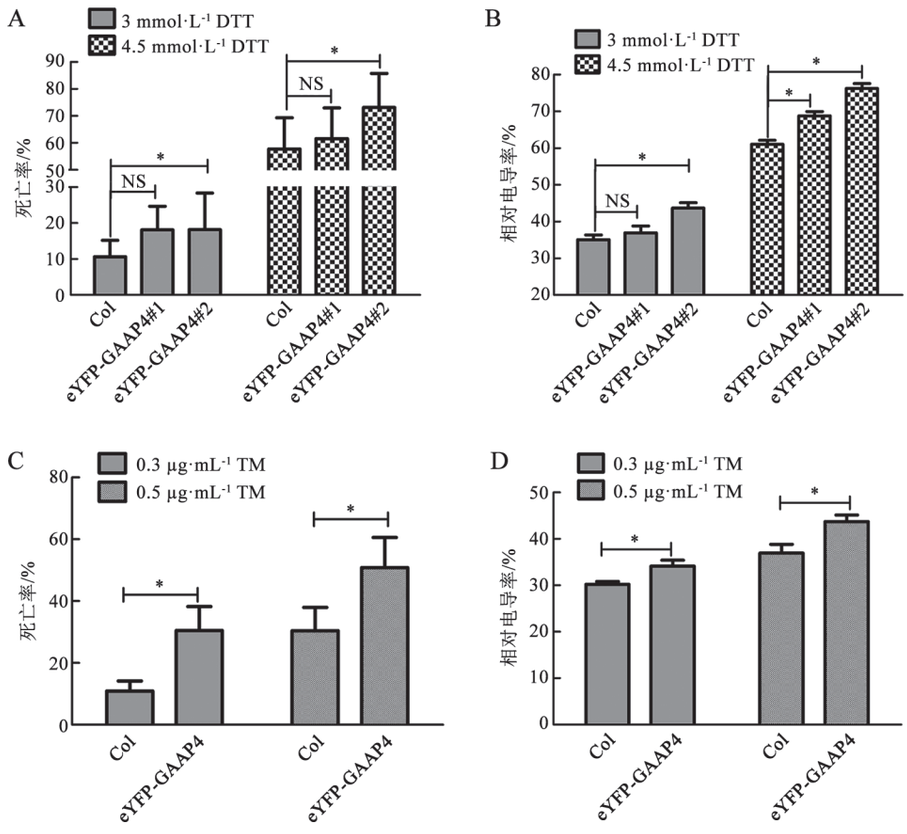

图5 eYFP-GAAP4过量表达增强幼苗的ER胁迫敏感性

Fig.5 Overexpressing eYFP-tagged GAAP4 increased the sensitivity of seedlings to ER stress

A~B: 生长3 d的幼苗转移至含DTT的培养基上生长7 d的植株死亡率(A)和电导率(B); C~D: 生长3 d的幼苗转移至含TM的培养基上生长10 d的植株死亡率(C)和电导率(D) (χ^2检验死亡率, *t*检验电导率, *: $P<0.05$, $n=5$)。

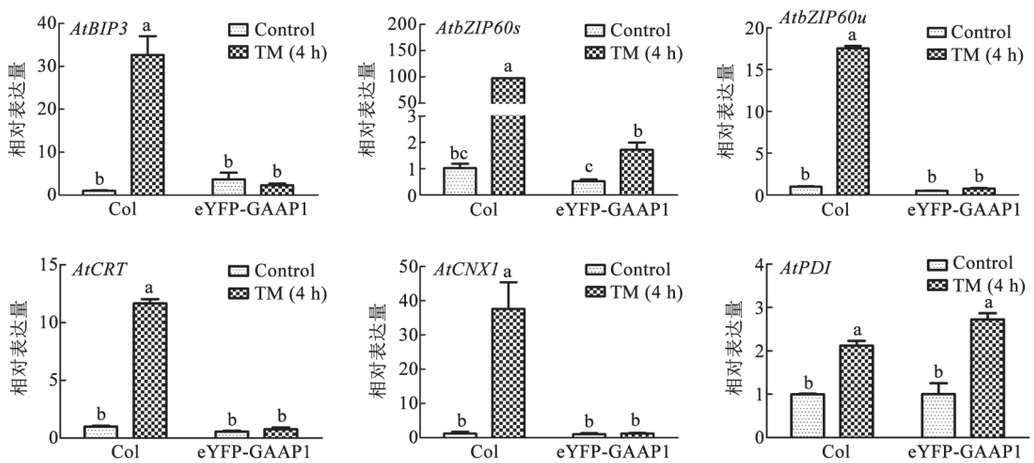

图6 eYFP-GAAP1过量表达削弱急性ER胁迫下UPR基因的表达

Fig.6 Overexpressing eYFP-GAAP1 reduced the induction of UPR genes upon acute ER stress assayed by qPCR

图中不同的字母表示显著性差异($P<0.05$)。

IRE1通路下游基因*bZIP60s*和*NAC103*、bZIP28通路下游基因*AtPDIL1*、*AtCNX1*和*AtHSP70*以及两条通路共有基因*AtBIP3* (Liu等2007; Mishiba等2013)的表达量。结果显示, eYFP-GAAP1和Col中*NAC103*的表达量变化幅度差异不大; 而TM处理12~36 h, eYFP-GAAP1中其他标记基因的表达量都低于Col。而与Col相比, eYFP-GAAP1中IRE1和bZIP28通路的表达模式并未受到影响(图7)。这些数据表明eYFP-GAAP1削弱了ER胁迫下UPR相关基因的诱导。

3 讨论

GAAPs是定位在内质网和高尔基体膜上的高度保守的细胞保护蛋白(Henke等2011), 拟南芥中有5个GAAPs家族成员。我们最近的研究发现GAAP1和GAAP3单突变以及双突变都提高了对于ER胁迫的敏感性, GAAP1和GAAP3过量表达都增强植株ER胁迫抗性(Guo等2018), 然而GAAP1和GAAP4的N端融合eYFP的过量表达转化子却增强幼苗的ER胁迫敏感性(图2~5)。这表明位于GAAPs-N端连接YFP标签会影响ER胁迫下GAAPs的功能。

在ER胁迫下, 细胞会激活未折叠蛋白响应通路(UPR)以缓解胁迫。在植物中已经发现两条UPR通路, 分别是IRE1A/B和bZIP28 (Liu等2007; Liu和Howell 2010; Mishiba等2013)。动植物细胞若无法缓解ER胁迫, 则会诱导PCD的发生(Lisbona等2009; Yang等2014)。GAAP1和GAAP3突变体在ER胁迫下的细胞死亡明显提高, 表明两者能够抑制ER胁迫诱导的细胞死亡。我们的研究表明, eYFP-GAAP1和eYFP-GAAP4的异源表达不仅增强ER胁迫下的细胞死亡(图4), 还抑制ER胁迫对于UPR信号通路的激活(图6和7)。保护性通路的削弱导致eYFP-GAAP1和eYFP-GAAP4植株对于ER胁迫高度敏感。显然, eYFP位于GAAP的N末端会影响蛋白的功能。在动物细胞中, 也有研究发现GFP标签位于BI-1的N端或C端对BI-1抑制细胞死亡以及维持Ca^{2+}平衡的功能具有不同的影响(Henke等2011)。综合这些研究结果表明, GFP或YFP位于N末端可能会改变BI-1、GAAPs的空间结构或细胞定位, 影响与其他蛋白因子的互作。而我们已有实验结果表明C末端有YFP标签的GAAP1功能与没有标签的蛋白功能类似(未发表数据), 因此, 在BI-1或GAAPs的功能研究中, 应将YFP或其他标签连接在蛋白质的C末端。

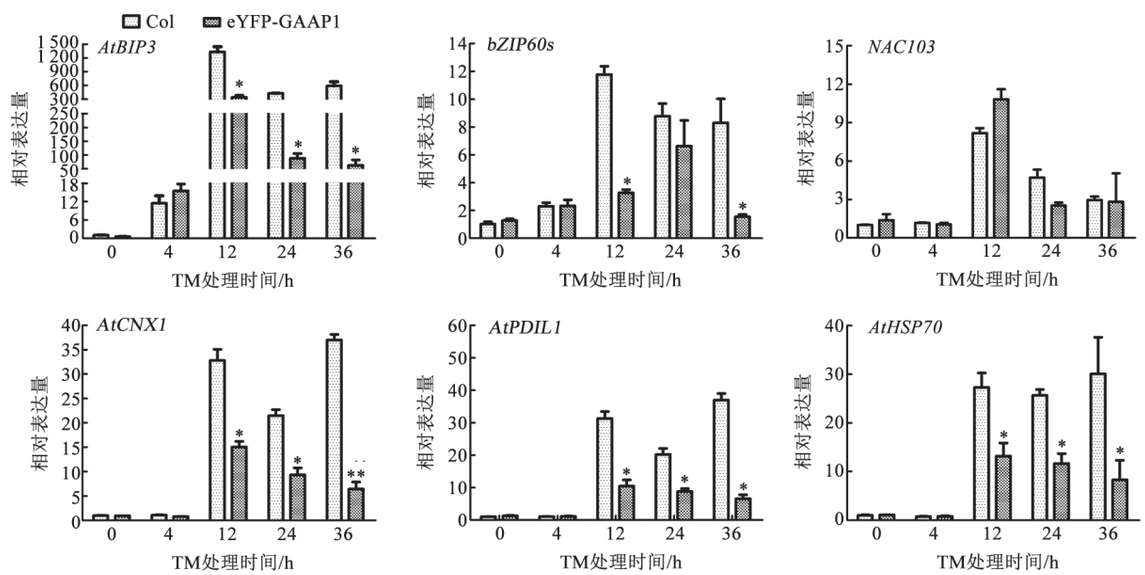

图7 eYFP-GAAP1的过量表达削弱ER胁迫持续过程中UPR基因的上调

Fig.7 Overexpressing eYFP-GAAP1 reduced the up-regulation of UPR gene over the time course of chronic ER stress

图中*: $P < 0.05$, **: $P < 0.01$。

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Ectopic expression of GAAPs tagged with eYFP at the N-terminal in *Arabidopsis* enhanced plant sensitivity to endoplasmic reticulum stress

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Abstract: Golgi anti-apoptotic proteins (GAAPs) are highly conserved throughout eukaryotes and GAAP resembles BAX inhibitor-1 with inhibiting apoptosis triggered by various stresses in mammalian cells. *Arabidopsis* GAAPs have also been showed inhibited cell death induced by endoplasmic reticulum (ER) stress by reverse genetic assay. Except for the mutants, the transgenic plants expressing the gene fused with different tags are usually generated to study the function and its mechanism. But it should be caution whether the tag affect the protein function firstly. In the present study, we generated *Arabidopsis* plants overexpressing GAAP1 or GAAP4 tagged with eYFP at their N-terminal respectively. The sensitivity of eYFP-GAAP1/GAAP4 and *gaap1* mutants to the ER-stress was tested. And the results showed that overexpressing eYFP-GAAP1/GAAP4 reduced the plant survival and enhanced cell death under ER stress, which was similar as *gaap1* mutants. The quantitative real time PCR analysis showed that overexpressing eYFP-GAAP1 inhibited the activation of unfolded protein response (UPR) signaling pathway genes upon acute or prolonged ER stress. These data suggested that the eYFP tag at the N-terminal of GAAPs affected its function in the resistance of ER stress and anti-programmed cell death.

Key words: *Arabidopsis thaliana*; GAAPs; ER stress; cell death; unfolded protein response

Received 2018-04-09 Accepted 2018-05-14

This work was supported by the National Natural Science Foundation of China (31670271) and the Shanghai Natural Science Program (15ZR1410900).

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