

# SAGP 基因克隆及反义表达载体的构建

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**[摘要]** 为了研究不同启动子驱动的反义SAGP抑制内源ADP-Glc PPase小亚基编码基因表达的效果, 创造适于外源基因表达的突变体, 利用RT-PCR方法, 由马铃薯块茎cDNA克隆获得ADP-Glc PPase小亚基编码基因(SAGP)的1 821 bp cDNA。核酸数据库检索和序列比对分析表明, 已克隆的SAGP基因cDNA推测编码607个氨基酸组成的多肽, 其中第312和411的天冬酰胺均突变为丝氨酸。以pCambia为基础, 分别构建了CaMV 35S和GBSSI块茎启动子驱动的小亚基cDNA反义结构植物表达载体。

**[关键词]** 腺苷二磷酸葡萄糖焦磷酸化酶(ADP-Glc PPase); 基因克隆; 启动子; 反义结构; 表达载体

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## Cloning of SAGP gene and constructing of antisense transformation vectors

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**Abstract:** To compare effect of expression repression antisense SAGP driven by two promoters and subsequently to create potato mutation of tuber starch deficiency, a full length of cDNA encoding small subunit of ADP-Glc PPase (SAGP) was cloned and sequenced through RT-PCR with tuber of potato cultivar Zheng 1011. The sequence BLAST and alignment showed that SAGP of the 1 821 bp cDNA encodes a deduced peptide composed of 607 amino acids, and two mutations, N312S and N411S, occurred on the peptide. With known vector pCambia for plant transformation, two antisense recombinant vectors, pS-SAGP and pG-SAGP in which SAGP were driven by promoters of 35S and GBSSI respectively, were constructed.

**Key words:** ADP-Glc PPase; gene cloning; promoter; antisense structure; transformation vector

腺苷二磷酸葡萄糖焦磷酸化酶(ADP-glucose pyrophosphorylase ADP-Glc PPase)催化磷酸葡萄糖和ATP合成ADP-葡萄糖, 后者是多数细菌和高等植物糖原和淀粉生物合成的葡萄糖活化供体; 大肠杆菌的ADP-Glc PPase是由*glgC*基因编码的同源四聚体<sup>[1-5]</sup>; 高等植物的ADP-Glc PPase是分别由不同的两个基因家族编码的大小亚基形成的异源四聚体, 因其对环境因素(如昼夜周期等)和代谢中间产物敏感而成为高等植物淀粉合成的限速酶, 以往研究已分别

从小麦、拟南芥和马铃薯等植物中克隆了不同的ADP-Glc PPase大小亚基编码基因<sup>[6-11]</sup>。

到目前为止, GenBank中有记录的马铃薯ADP-Glc PPase小亚基编码序列cDNA有4个, gDNA有2个; 大亚基编码序列cDNA有4个, gDNA有2个<sup>[12-13]</sup>。从马铃薯块茎中分离纯化的ADP-Glc PPase由2个51 ku的大亚基和2个50 ku的小亚基组成<sup>[9, 14-15]</sup>; 在其后对包括马铃薯在内的不同植物大小亚基重要氨基酸和结构域的点突变研

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究中发现,ADP-Glc PPase 大亚基主要起调节作用,小亚基主要起合成作用<sup>[16-19]</sup>。另外,按照激活和抑制因子的不同,将ADP-Glc PPase 分为9类,其中激活和抑制大肠杆菌ADP-Glc PPase 的因子不存在于高等植物中,反之亦然<sup>[20]</sup>。

M ller-Rober 等<sup>[21]</sup>构建了由35S启动子驱动的马铃薯ADP-Glc PPase 小亚基编码基因(*SAGP*)反义结构并转化马铃薯,结果转基因块茎和叶片内淀粉含量显著降低,块茎干物质含量降低30%,而蔗糖和葡萄糖积累增加。因本研究所用35S启动子是非特异表达启动子,因此其转基因个体在叶片淀粉积累改变的前提下,无法确定块茎中淀粉含量的变化是由于叶片运输到地下组织蔗糖的减少造成的,还是由于块茎中ADP-Glc PPase 的变化造成的;如果两者兼而有之,那么完全停止块茎中ADP-Glc PPase 活性,能否关闭或继续降低淀粉合成,尚有待于进一步研究。本研究旨在分别构建35S和*GBSSI*启动子驱动的反义*SAGP*植物表达载体,为比较研究不同组织器官淀粉积累的方式奠定基础。

## 1 材料与方法

### 1.1 *SAGP* 基因cDNA 克隆

以新鲜马铃薯品系郑1011为材料,用氯化锂沉淀法提取块茎RNA,反转录合成*SAGP* cDNA 的第一链。反转录反应体系中包含0.1 μg RNA, 1 μL Oligo (dT), 2 μL dNTP, 0.5 μL RNase 抑制因子, 0.5 μL 5×缓冲液, 0.5 μL AMV 反转录酶,加去离子无菌水至20 μL。在42℃恒温水浴中保温1 h,之

后在冰浴中冷却2 min。以反转录产物为模板,上游引物为APF: 5'-GCA ATC ACA CTC TAC CAC A-3';下游引物为APR: 5'-TTT TTT TTT GAG TAA TTT ATT T-3'。在Eppendorff Master Cycler 进行聚合酶链式反应(PCR)。反应体系组成为: 5 μL 10×缓冲液, 4 μL dNTP, 上下游引物各4 μL, 10 μL cDNA, 30 μL MgCl<sub>2</sub>, 0.5 μL *Taq* DNA 聚合酶(Takara), 20.5 μL ddH<sub>2</sub>O。反应程序为: 95℃预变性4 min, 其后35个循环按照94℃变性45 s, 46℃退火1 min, 72℃延伸2 min进行,而后72℃延伸10 min, 4℃终止反应。

PCR 产物纯化按试剂盒(Wizard DNA purification system)说明进行,用pGEM-T-Easy 为克隆载体进行PCR 产物与载体连接,连接体系由1 μL pGEM-T-Easy, 1 μL PCR 产物, 5 μL 10×缓冲液, 1 μL *T<sub>4</sub>* 连接酶, 2 μL ddH<sub>2</sub>O 组成, 4℃下过夜。连接产物转化感受态大肠杆菌JM 109, 其在表面涂有10 μL 24 mg/L 的异丙基-β-D-硫代半乳糖苷(IPTG)和40 μL 20 mg/mL X-gal; 在含有1 μL/mL 氨苄青霉素15 mL 的LB 平板培养基上筛选阳性菌落,用Mini prep 方法提取重组质粒,在pGEM-T-Easy 载体MCS 和*SAGP* 序列内选择酶切位点,对重组质粒进行酶切;在含有溴乙烷的琼脂糖凝胶上进行电泳,电泳条件为75 V 电压,电泳时间60 min。本试验中除另外指出外,其他电泳分析均与此条件相同。分子质量标准用DL 2000(Takara),然后测序确认克隆序列的正确性,序列测定由北京华大中生公司完成。

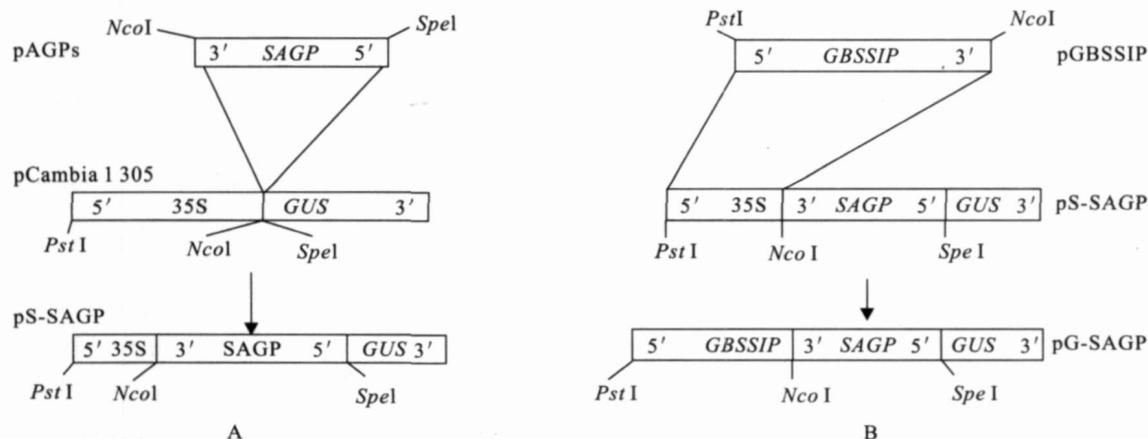


图1 反义*SAGP* 表达载体构建示意

A. pS-SAGP 表达载体; B. pG-SAGP 表达载体; GBSSIP. *GBSSI* 启动子; 35S 烟草花叶病毒35S 启动子

Fig 1 Schematic diagram of the vector containing antisense *SAGP*

A. Construct of pS-SAGP expressing vector; B. Construct of pG-SAGP expressing vector; GBSSIP. *GBSSI* promoter; 35S 35S promoter from CaMV

## 1.2 pS-*SAGP* 反义载体的构建

为了利用pCambia-1305-1中T-DNA区域内的35S启动子,将*SAGP*基因cDNA反向连接在启动子的3'端(图1A),用*Nco*I/*Spe*I分别酶切p-*SAGP*和pCambia-1305-1(CAMBIA),纯化*SAGP*和PCambia-1305-1片段,连接反应体系由1 μL *T*<sub>4</sub>连接酶,6 μL纯化的*SAGP*片段,1 μL纯化的线性PCambia-1305-1载体,1 μL连接反应缓冲液和1 μL ddH<sub>2</sub>O组成,16 h过夜。连接产物转化感受态大肠杆菌JM 109,在含50 mg/mL卡那霉素的LB培养基平板上筛选阳性菌落。通过miniprep提取重组质粒,分别用*Hind*III和*Eco*R I单酶切进行鉴定。

## 1.3 pG-*SAGP* 反义载体的构建

为了用*GBSSI*启动子驱动*SAGP*,将pS-*SAGP*中的35S启动子用*GBSSI*启动子代替,如图1B所示。首先用*Nco*I/*Pst*I分别酶切pS-*SAGP*和p*GBSSI*P载体(本实验室保存),纯化回收无35S启动子的线性pS-*SAGP*和*GBSSI*启动子。连接反应体系同1.2中相应部分。转化感受态大肠杆菌JM 109,在含有Kan的LB培养基平板上筛选阳性菌落,少量制备重组质粒。在PCambia-1305-1多克隆位点,*GBSSI*启动子和*SAGP*内选酶切位点,分别用*Hind*III和*Eco*R I单酶切进行鉴定。

## 2 结果与分析

### 2.1 *SAGP* cDNA 的克隆

对连接了*SAGP* cDNA的阳性菌落进行Miniprep,获得了重组质粒。图2表明,对重组质粒进行*Pst*I和*Nco*I双酶切后得到2个片段,一个是

包含*SAGP* cDNA的约1.8 kb条带,一个为线性化pGEM-T-Easy载体;*Hind*III单酶切得到2个片段,一个为1.2 kb条带,另一个为约3.8 kb的线性化载体;*Pst*I和*Bgl*II酶切得到一个0.5 kb片段和一个约4.3 kb的载体;以上酶切结果与预期相符。为进一步证实获得的*SAGP* cDNA序列的正确性,对克隆的*SAGP* cDNA进行测序,结果表明,*SAGP* cDNA序列5'和3'分别连接在pGEM-T-Easy多克隆位点的SP6及T7端,该*SAGP* cDNA序列全长1821 bp,GenBank登记号为DQ297414。而后在核酸数据库中对序列进行BLAST和序列比对,结果表明,已克隆的cDNA与数据库中检索到的马铃薯4个ADP-Glc PPase小亚基编码基因序列,有99.9%的一致性,预测的小亚基多肽链中有2个突变位点,即第312和411的天冬酰胺均突变为丝氨酸(图3)。将*SAGP* cDNA与pGEM-T-Easy重组质粒命名为pAGPs。

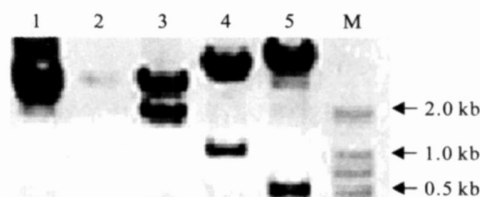


图2 重组质粒pAGP酶切电泳结果

1. 重组载体; 2. pGEM-T-Easy; 3. *Pst*I-*Nco*I酶切;  
4. *Hind*III酶切; 5. *Pst*I-*Bgl*II酶切; M. 分子量标准

Fig. 2 Digestion analysis of pAGP with various enzymes

1. pAGP without digestion; 2. pGEM-T-Easy; 3. pAGP with  
*Pst*I/*Nco*I digestion; 4. pAGP with *Hind*III digestion;  
5. pAGP with *Pst*I/*Bgl*II digestion; M. molecular weight mark

|        |                                                                                      |
|--------|--------------------------------------------------------------------------------------|
| Nakata | GIYVISKDVMLNLLRDKFPGANDFGSEVIPGATSLGMRVQAYLYDGYWEDIGTIEAFYNANLGITKKPVPDFSFYDRSAPIYTQ |
| Muller | GIYVISKDVMLNLLRDKFPGANDFGSEVIPGATSLGMRVQAYLYDGYWEDIGTIEAFYNANLGITKKPVPDFSFYDRSAPIYTQ |
| Jardin | GIYVISKDVMLNLLRDKFPGANDFGSEVIPGATSLGMRVQAYLYDGYWEDIGTIEAFYNANLGITKKPVPDFSFYDRSAPIYTQ |
| YXL    | GIYVISKDVMLNLLRDKFPGANDFGSEVIPGATSLGMRVQAYLYDGYWEDIGTIEAFYNANLGITKKPVPDFSFYDRSAPIYTQ |
| Liu    | GIYVISKDVMLNLLRDKFPGANDFGSEVIPGATSLGMRVQAYLYDGYWEDIGTIEAFYNANLGITKKPVPDFSFYDRSAPIYTQ |
|        | 312                                                                                  |
| Nakata | PRYLPPSKMLDADVTDSVIGEGCVIKNCKIHHSVGLRSCISEGAIIEDSLLMGADYYETDADRKLLAAKGSVPIGIGKNCHIKR |
| Muller | PRYLPPSKMLDADVTDSVIGEGCVIKNCKIHHSVGLRSCISEGAIIEDSLLMGADYYETDADRKLLAAKGSVPIGIGKNCHIKR |
| Jardin | PRYLPPSKMLDADVTDSVIGEGCVIKNCKIHHSVGLRSCISEGAIIEDSLLMGADYYETDADRKLLAAKGSVPIGIGKNCHIKR |
| YXL    | PRYLPPSKMLDADVTDSVIGEGCVIKNCKIHHSVGLRSCISEGAIIEDSLLMGADYYETDADRKLLAAKGSVPIGIGKNCHIKR |
| Liu    | PRYLPPSKMLDADVTDSVIGEGCVIKNCKIHHSVGLRSCISEGAIIEDSLLMGADYYETDADRKLLAAKGSVPIGIGKNCHIKR |
|        | 411                                                                                  |
| Nakata | AIIDKNARIGDNVKKIINKDNVQEAARETDGYFIKSGIVTVIKDALIPSGIII                                |
| Muller | AIIDKNARIGDNVKKIINKDNVQEAARETDGYFIKSGIVTVIKDALIPSGIII                                |
| Jardin | AIIDKNARIGDNVKKIINKDNVQEAARETDGYFIKSGIVTVIKDALIPSGIII                                |
| YXL    | AIIDKNARIGDNVKKIINKDNVQEAARETDGYFIKSGIVTVIKDALIPSGIII                                |
| Liu    | AIIDKNARIGDNVKKIINKDNVQEAARETDGYFIKSGIVTVIKDALIPSGIII                                |

图3 研究所得*SAGP* cDNA预测的编码多肽序列与已报道序列比对

Nakata, Muller, Jardin, Liu 分别代表该*SAGP*氨基酸序列对应基因相关文章作者, YXL为本研究分离获得的*SAGP*部分序列

Fig. 3 A alignment between partial *SAGP* peptide sequences reported and obtained in this study

Nakata, Muller, Jardin, YXL, Liu represents author's first name of publications, by whom *SAGP* sequences of amino acids were predicted with gene he cloned

## 2.2 反义SA GP 表达载体pS-SA GP 的构建

按照1.2描述的方法,对重组质粒进行酶切,电泳结果见图4。图4表明,预期的目的条带出现在电泳胶中,这说明以CaMV 35S 为启动子的反义SA GP 表达载体构建正确,将重组质粒命名为pS-SA GP。

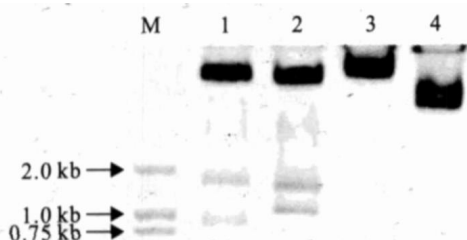


图4 pS-SA GP 反义表达载体酶切电泳结果

M. 分子量标准; 1 *EcoRI* 酶切; 2 *HindIII*酶切;  
3 pS-SA GP; 4 pCambia-1305-1

Fig 4 Digestion analysis of pS-SA GP

M. molecular weight mark; 1- 2 digested results  
with *EcoRI* / *HindIII* respectively;  
3 pS-SA GP; 4 pCambia-1305-1

## 2.3 反义SA GP 表达载体pG-SA GP 的构建

用*GBSSI* 启动子替换pS-SA GP 中的35S 启动子, 替换后重组载体序列中存在3个*EcoRI* 酶切位点和3个*HindIII*酶切位点。预期通过*EcoRI* 酶切得到1.0 kb, 1.5 kb 和线性载体3个片段, 通过*HindIII*酶切得到0.9 kb, 1.0 kb 和线性载体3个片段。由图5可知, 重组载体酶切和电泳后, 在琼脂糖电泳凝胶上观察到的条带与以上期望结果相同, 表明*GBSSI* 启动子驱动的SA GP 反义表达载体构建正确, 将此重组载体命名为pG-SA GP。

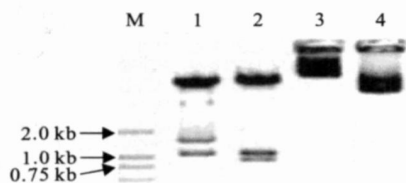


图5 pG-SA GP 反义表达载体酶切电泳结果

M. 分子量标准; 1 *EcoRI* 酶切; 2 *HindIII*酶切;  
3 pG-SA GP; 4 pCambia1305-1

Fig 5 pG-SA GP digested with various enzymes

M. molecular weight mark; 1- 2 digestion  
with *EcoRI* / *HindIII* respectively;  
3 pG-SA GP; 4 pCambia1305-1

## 3 讨论

尽管目前RNA 干扰途径正在替代反义RNA 抑制技术, 而成为基因功能敲除的重要手段之一, 但因淀粉合成过程中的相关基因序列具有不同程度的相似性等原因, 到目前为止尚未发现合适的ADP-Glc PPase 小亚基特异的小片段RNA。

保持代谢“源”组织和“库”组织间代谢中间产物的平衡, 是“库”组织淀粉积累的重要条件, 体内条件下两组织间的平衡受多种因素的影响, 很难获得在成熟期淀粉零积累的边际条件; 同时, 对于多倍体植物(如马铃薯)而言, 很难发现无淀粉积累的突变体, 这些给研究“源”和“库”组织代谢中间产物的动态平衡, 带来了很大困难。即使是用外源ADP-Glc PPase 编码基因在马铃薯体内表达, 进行淀粉生物合成代谢调控研究, 也因为内源ADP-Glc PPase 编码基因的存在而无法分辨是外源还是内源基因表达的结果。为此, 本试验分别构建了35S 和*GBSSI* 启动子驱动的反义SA GP 植物表达载体, 旨在通过体内转化形成整株和块茎淀粉生物合成缺陷突变体, 分别以整株淀粉合成缺陷突变体和淀粉合成功能齐全的野生型为对照, 研究代谢“源”组织和“库”组织淀粉积累的动态平衡, 同时也为外源ADP-Glc PPase 编码基因在马铃薯体内的表达及淀粉品质的改良提供基本材料。

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