拟南芥中人工启动子GWSF转录特性及乙烯诱导活性分析

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摘要: 理想的病原诱导型启动子可用来精确调控抗病基因的时空表达, 消除转基因中的副作用。选用Gst1-box、W-box、S-box、F-box元件和CaMV minimal 35S启动子设计长度为374 bp的人工启动子GWSF。用GWSF替代pBI121中调控*gus*基因的CaMV 35S启动子后, 将重组质粒导入农杆菌GV3101, 并通过花序浸泡法得到转GWSF:*gus*拟南芥。以T_4代植株为材料, GUS染色发现GWSF本底表达低, 受疫霉菌、青枯菌及抗病信号分子水杨酸的诱导, 但不受非致病菌大肠杆菌和逆境激素脱落酸(ABA)的诱导。序列分析发现, GWSF含有乙烯应答元件(ERE)。用乙烯处理T_4代转基因植株, 通过实时荧光定量PCR (qPCR)检测GWSF的乙烯诱导活性。当乙烯浓度为0.2 mmol·L^{-1}时, GWSF转录活性最高, 达到CaMV 35S的11.33倍。结果表明, GWSF是较为理想的植物病原和乙烯诱导型启动子, 具有本底表达低、诱导因子广、启动表达快、诱导效率高等优点, 可用于植物转基因抗病育种。

关键词: 人工启动子; GWSF; 乙烯

通过防御基因的过表达提高作物抗性常因过多的能量耗费影响转基因作物的农艺性状, 因此选用合适的启动子在转基因抗病中非常重要(Werner等2011)。天然启动子在表达强度和特异性等方面存在一定的局限性(Sahoo等2014)。理想的病原物诱导型启动子应该具有诱导因子广、启动表达快、诱导效率高、本底活性低且不受损伤诱导等特点(Huang等2017)。

研究发现, Gst1-box、W-box、S-box、F-box等顺式作用元件有诱导表达活性, 且有诱导因子广、本底活性低、不受损伤诱导等特点(Mazarei等2008; Mohr等2010)。由于同一启动子元件在不同植物种类中相对保守(Schlabach等2010), 组合这些元件的二聚体及CaMV (cauliflower mosaic virus) minimal 35S启动子(Benfey等1990)有望得到理想的人工病原物诱导启动子(synthetic pathogen-inducible promoter, SPIP), 实现抗病基因在时空上的精确表达(彭舒等2011)。

植物生长发育过程中, 会遭受多种病原菌的侵扰, 同时环境中又存在多种非致病菌以及各种非生物逆境。受致病菌侵染时植物常会产生水杨酸(Fu等2012)和乙烯(Yang等2017)来增强抗性。非生物逆境下植物常积累脱落酸(abscisic acid, ABA) (Li等2012b)来应答胁迫。理想的病原物诱导型启动子应只受致病菌侵染的诱导, 而不受非生物逆境和非致病菌的诱导。

我们的前期研究从8个SPIP中筛选出具备本底表达低且受疫霉菌、青枯菌及水杨酸等诱导的GWSF (Huang等2017)。本实验以转GWSF:*gus*拟南芥T_4代植株为材料, 进一步探讨了人工启动子GWSF转录特性及乙烯诱导活性, 为GWSF在抗病基因工程中的应用及其优化改造提供参考。

1 材料与方法

1.1 材料与试剂

拟南芥(*Arabidopsis thaliana* L.) Columbia (Col-0)生态型, 培养于光照培养箱。培养条件为: 光照强度300 μmol·m^{-2}·s^{-1}, 光周期14 h光照/10 h黑暗, 相对湿度70%~80%。大肠杆菌(*Escherichia coli*) DH5α、疫霉菌(*Phytophthora capsici*)、青枯菌(*Ralstonia solanacearum*)为本实验室纯化保存菌株。UNIQ-10柱式TRIzol总RNA抽提试剂盒、卡那霉素(Kam)、乙烯利、水杨酸、GLUC等购自上海生工。反转录试剂盒(PrimeScript RT reagent kit with gDNA eraser)和实时荧光定量PCR (real-time quantitative PCR, qPCR)试剂(SYBR Premix Ex Taq^{TM} II)购自宝生物公司。

1.2 人工启动子设计和载体构建

4种具病原诱导表达元件Gst1-box (5′-TTC-TAGCCACCAGATTTGACCAAAC-3′; Malnoy等

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2006)、W-box (5′-TTATTCAGCCATCAAAAGTTGACCAATAAT-3′; Wang等1998)、S-box (5′-CAGCCACCAAAGAGGACCCAGAAT-3′; Kirsch等2000)、F-box (5′-TTGTCAATGTCATTAAATTCAAACATTCAACGGTCAATT-3′; Heise等2002)被用来组合SPIP。先用6 bp的插入序列(ACTAGA)将相同元件连成二聚体, 再用10 bp的连接序列(GAAGATAATC) (Cazzonelli和Velten 2008)按Gst1-box、W-box、S-box、F-box的顺序将各二聚体连成八聚体, 最后用3个串联的10 bp的连接序列将八聚体和CaMV minimal 35S启动子连接成长为374 bp的GWSF人工启动子。在网站http://www.softberry.com/ (TSSP/Prediction of PLANT Promoters)进行启动子序列分析。在GWSF的5′和3′两端分别引入*Hin*dIII和*Bam*HI位点, 交上海生工合成并克隆到pUC19载体。通过酶切、连接后转化, 用GWSF替代pBI121的CaMV 35S片段, 将*gus*基因置于GWSF调控之下, 导入到农杆菌(*Agrobacterium tumefaciens*) GV3101用于转化。

1.3 遗传转化及筛选

用农杆菌介导的花序浸泡法(Chen等1994)转化野生型拟南芥, 分别收集17株T_1代种子。将种子干燥后点种在含50 mg·L^{-1} Kam的1/2MS固体培养基。2周后将绿苗种于蛭石基质, 置于气候箱培养。T_2代种子成熟后取叶片提DNA进行PCR检验。实验所需引物见表1。再用Kam筛选T_2代种子, 统计绿苗和白化苗的比例, 将绿苗与白化苗的比例最接近3:1的株系视为单拷贝转基因子代, 转移栽种于蛭石基质, 于气候箱培养得到T_3代种子。重复Kam筛选和PCR检验, 获得T_4代植株用于GUS染色和qPCR。

1.4 诱导处理及GUS染色

培养至6周的拟南芥用大肠杆菌、青枯菌、疫霉菌、水杨酸、ABA、乙烯利等诱导。大肠杆菌、青枯菌、疫霉菌孢子用无菌水配制成1×10^6 CFU (colony-forming unit)的悬液, 喷施到植株表面, 每株2 mL, 诱导时间6 h。水杨酸浓度2 mmol·L^{-1}, ABA浓度0.2 mmol·L^{-1}, 喷施到植株表面, 每株2 mL, 诱导时间6 h。乙烯利诱导处理如下: 配制40%的乙烯利溶液50 mL (定容时加1 mmol·L^{-1} HCl 2 mL使pH小于4.0)。在22.4 L的塑料桶中放入3钵拟南芥, 每钵3株, 再在桶中放入盛有200 mL 1% (*m*/*V*) $NaHCO_3$的锥形瓶。向锥形瓶中分别加入乙烯利溶液0、0.375、0.75、1.5、3、6 mL, 使乙烯利充分释放后对应的浓度分别为0、0.05、0.1、0.2、0.4、0.8 mmol·L^{-1}。密封塑料桶, 处理时间12 h。受伤处理为: 用无针头的1 mL注射器摩擦莲座叶上表皮至破损, 尽量不损伤到叶肉组织。受伤处理后喷无菌蒸馏水, 每株2 mL, 诱导时间12 h。以上各处理3个重复, 时间到后, 剪取莲座叶参照Jefferson等(1987)的方法进行GUS染色。

1.5 qPCR检测

剪取约30 mg叶片, 用UNIQ-10柱式TRIzol总RNA抽提试剂盒提取总RNA, 用NanoDrop 2000C检测总RNA质量。以总RNA为模板, 用反转录试剂盒(PrimeScript RT reagent kit with gDNA eraser)反转录得cDNA。用Pfaffl (2001)相对定量法检测*gus*基因的转录, 计算GWSF转录活性。内参基因用拟南芥*EF1-α* (AT5G60390), 实验所需引物见表1。反应体系均为25 μL, 含2×SYBR Premix Ex Taq™ II (TliRNase H Plus) 12.5 μL、10 μmol·L^{-1}上下游引物各1 μL、ddH$_2$O 8.5 μL、cDNA模板2 μL。设转CaMV 35S:*gus*基因中CaMV 35S的转录活性为1, 计算*gus*基因的相对表达值, 推知GWSF转录活性。

表1 PCR和qPCR所用的引物

Table 1 Primers used for PCR and qPCR

基因名称	正向引物序列(5′→3′)	反向引物序列(5′→3′)
GWSF (PCR)	TTGAAGATA ATCCAGCCACCA	AGCGTGTCCTCTCCAAATGA
NPT II (PCR)	GAGGCTATTCGGCTATGACTG	ATCGGGAGCGGCGATACCGTA
gus (PCR)	ACACCGATACCATCAGCG	TCACCGAAGTTCATGCCAGT
EF1-α (qPCR)	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA
gus (qPCR)	CTGATAGCGCGTGACAAAAA	GGCACAGCACATCAAAGAGA

2 实验结果

2.1 诱导因子分析

诱导性病原物种类广是理想病原诱导启动子的特征之一。用不同类型诱导因子处理培养6周的转GWSF:*gus*拟南芥T_4代植株后进行GUS染色, 结果显示, GWSF符合本底表达低(图1-C)的要求, 能受致病菌青枯菌(图1-E)、疫霉菌孢子(图1-F)及抗病相关激素水杨酸(图1-G)的诱导。非致病菌大肠杆菌(图1-D)及非生物逆境应答激素ABA (图1-H)对GWSF无明显诱导效果。GUS染色实验发现, 密闭环境(塑料瓶盖密封的果酱瓶)中生长的转GWSF:*gus*拟南芥本底表达高, 而种植在营养钵中的拟南芥本底表达低, 推测密闭环境下拟南芥自身释放积累的乙烯可能影响GWSF的本底表达。用Softberry软件的TSSP分析GWSF, 发现Gst1-box中含有乙烯应答元件(ethylene response element, ERE) (GATTTGACCAA)。喷施乙烯利证实GWSF受乙烯诱导(图1-I), 而非密闭环境(营养钵)下, GWSF的本底表达低(图1-C)。另外, 转GWSF:*gus*拟南芥受青枯菌、疫霉菌孢子、水杨酸和乙烯诱导后, 莲座叶染色深, 薹上叶染色浅或不着色, 说明GWSF转录活性受组织细胞位置的影响。

2.2 乙烯诱导活性分析

经不同浓度乙烯和受伤处理12 h后, 设CaMV 35S的转录活性为1, 用相对定量的Pfaffl (2001)的方法计算乙烯浓度为0、0.05、0.1、0.2、0.4、0.8 mmol·L^{-1}时, GWSF的转录活性分别为0.17、1.72、4.89、11.33、10.54、9.51 (图2)。乙烯浓度在0~0.2 mmol·L^{-1}范围内, 随浓度升高, GWSF转录活性逐渐增强, 乙烯浓度为0.2 mmol·L^{-1}时转录活性最高; 乙烯浓度在0.2~0.8 mmol·L^{-1}, 随浓度升高, GWSF转录活性逐渐减弱。可见, GWSF对乙烯诱导的响应与乙烯浓度密切相关。为了评估伤乙烯对GWSF转录活性的影响强度, 对转GWSF:*gus*拟南芥进行受伤处理, qPCR检测发现, 受伤处理后GWSF转录活性为0.20, 略高于本底, 但受伤处理与本底间无显著性差异, 说明一定程度的机械损伤不会引起GWSF转录活性的显著增强。

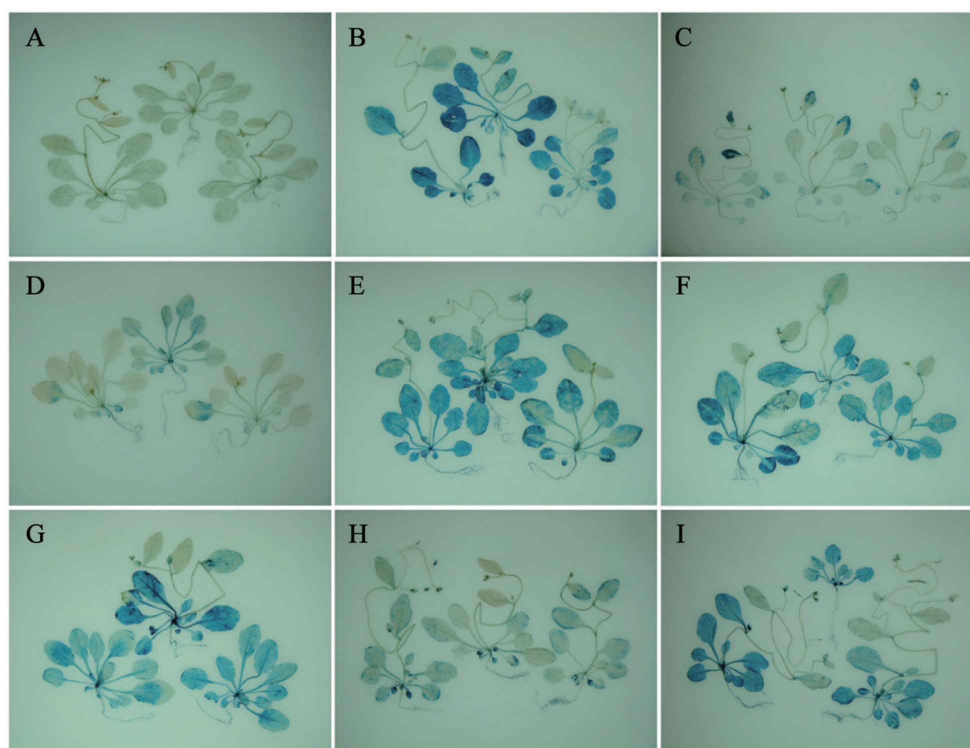

图1 转基因拟南芥中启动子GWSF的GUS染色

Fig.1 Histochemical localization of GUS activity in GWSF:*gus* transgenic *A. thaliana* plants

A: 野生型; B: CaMV 35S:*gus*的组成型表达; C: 本底表达; D: 非致病菌大肠杆菌诱导; E: 青枯菌诱导; F: 疫霉菌孢子诱导; G: 水杨酸诱导; H: ABA诱导; I: 乙烯诱导。

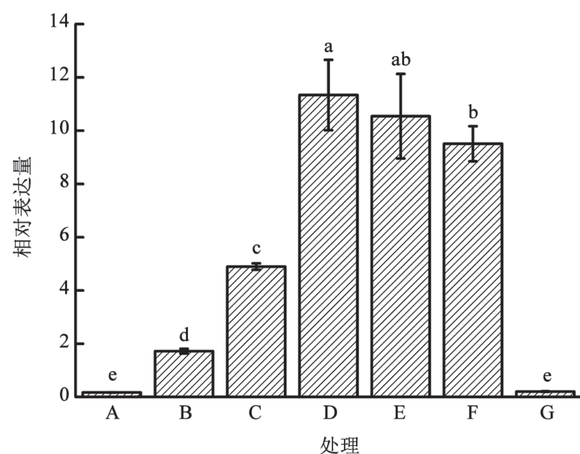

图2 不同浓度乙烯诱导下转GWSF:*gus*拟南芥中*gus*相对表达水平的qPCR分析

Fig.2 qPCR analysis of relative *gus* expression in GWSF:*gus* transgenic *A. thaliana* plants responsing to different ethylene concentrations

A: 对照(无菌水处理); B、C、D、E、F: 分别用0.05、0.10、0.20、0.40、0.80 mmol·L^{-1}乙烯诱导; G: 受伤处理。图中各处理间小写字母不同表示差异显著(P<0.05)。

3 讨论

转基因抗病中常利用强启动子促进抗病相关基因的表达, 增强植株抗病性。抗病基因的高效表达虽然能提高植物的抗病力, 但抗病基因的持续高水平表达会使植株因能量过多耗损、生长缓慢、畸变, 甚至死亡(Chen和Chen 2002)。众多转基因抗病策略中, 利用病原物诱导型启动子精确控制目的基因的表达, 将抗性反应限制在受感染时和被侵染位点可能最为有效(Sarah等2005a, b)。虽然天然启动子在表达强度和特异性等方面存在一定的局限性(Mehrotra等2011), 但同一启动子元件在不同植物种类中相对保守(Schlabach等2010), 组合这些元件有望得到理想的SPIP (Venter 2007)。

我们前期研究将Gst1-box、W-box、S-box、F-box四种元件进行排列组合, 设计出8个长度仅为374 bp的SPIP, 筛选得到的GWSF具备本底表达低, 受疫霉菌、青枯菌及水杨酸诱导等优点(Huang等2017)。本实验以转GWSF:*gus*拟南芥T_4代植株为材料, 深入探讨GWSF的转录特性。GUS染色结果显示, 转GWSF:*gus*拟南芥遗传稳定性好, T_4代植株中GWSF能保持本底表达低, 受疫霉菌、青枯菌和水杨酸诱导的优点。同时, 非致病菌大肠杆菌、ABA和受伤处理对GWSF无明显诱导效果。结果表明, 在本研究供试因子中, GWSF只受致病菌侵染的诱导, 而不受非生物逆境、受伤和非致病菌的诱导, 具备理想病原诱导启动子的特性。

使用Softberry软件的TSSP分析, GWSF含有ERE (GATTTGACCAA)。这一元件使得GWSF具有乙烯诱导活性。外源乙烯浓度为0.2 mmol·L^{-1}时GWSF转录活性最高, 达到CaMV 35S的11.33倍。GWSF对乙烯诱导的响应与乙烯浓度密切相关, 在0.2~0.8 mmol·L^{-1}范围内, 随乙烯浓度升高, GWSF转录活性反而减弱。乙烯调节种子萌发、营养生长、开花、成熟等多个代谢过程, 且常表现出与浓度相关的双重调控效应, 其调节反馈机制尚需进一步验证(Ning 2008; Li等2012a)。如果应用GWSF调控与诱导愈伤组织分化或芽分化相关基因的表达, 则可在组织培养阶段应用外源性乙烯或通过愈伤组织块自身释放乙烯的诱导启动基因表达, 起到促进再生, 提高植物转基因的成功率。

总之, GWSF长度小(374 bp), 具有本底表达低、诱导因子广、启动表达快、诱导效率高等优点, 是较好的SPIP。以此为基础进一步改良, 有望得到更为理想的SPIP, 用于植物遗传改良。

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Transcriptional properties and transcriptional activities to ethylene of a synthetic pathogen-inducible promoter GWSF in *Arabidopsis thaliana*

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Abstract: Ideal pathogen-inducible promoters meet the demands for desired temporal and spatial regulation of transgenes with minimal side effects. GWSF, a synthetic pathogen-inducible promoter (SPIP), was designed with Gst1-box, W-box, S-box, F-box and CaMV 35S minimal promoter. GWSF was used to replace the wild-type CaMV 35S promoter in the plasmid pBI121 in order to control the expression of the *β-glucuronidase* (*gus*) gene. The transcriptional properties and transcriptional activities in response to ethylene of GWSF were evaluated by histochemical staining and real-time quantitative PCR (qPCR) in homozygous T_4 lines of transgenic *Arabidopsis thaliana*. GWSF had a low basal expression and responded to *Ralstonia solanacearum*, *Phytophthora* capsici and salicylic acid, but did not respond to abscisic acid and *Escherichia coli*. GWSF could be induced by ethylene for an ethylene response element in its sequence. At a concentration of 0.2 $mmol \cdot L^{-1}$ ethylene, the transcriptional activities reached to the peak that was 11.33 times as high as the wild-type CaMV 35S promoter. The results indicate that GWSF is an ideal SPIP with the advantages of a low background expression, a wide range of inducing pathogens, rapid responses and efficient transcriptional activities. It can be potentially improved further to apply to plant genetic engineering for disease resistance.

Key words: synthetic pathogen-inducible promoter (SPIP); GWSF; ethylene

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