西瓜果实特异启动子 WSP 功能区域的初步定位

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摘 要 西瓜 AGPase 的大亚基基因 *wml*1 的 5′端上游 1573bp 序列,是一个果实特异性启动子(命名为 WSP)。根据 WSP 内部酶切位点,获得了 3 个不同 5′端缺失的启动子片段(长分别为 1201bp、898bp、795bp),并构建成植物瞬间表达载体,与含 WSP 的瞬间表达载体一起用基因枪的方法转入西瓜叶、茎、花及不同发育期果实中。瞬时表达结果表明,1573bp、1201bp、898bp 的片段均能指导 GUS 基因在西瓜果实和花中特异性表达,但是表达强度和表达时期有所不同,795bp 的片段不能指导 GUS 基因表达。推测在 180bp-551bp 之间可能存在促进外源基因在果实发育后期表达的顺式作用元件,而果实特异调控区域可能位于 854bp-957bp 之间。

关键词 西瓜,果实特异性,启动子,瞬时表达

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ADP-葡萄糖焦磷酸化酶(AGPase)是植物淀粉生物合成途径中的关键酶之一,催化 ADP-葡萄糖的合成。AGPase 是一个异型四聚体,由两个大亚基和两个小亚基构成[1]。在高等植物中,小亚基在所有器官中都表达,而大亚基的表达具有组织特异性[2-4]。淀粉是植物中最重要的碳的贮藏形式,主要在贮藏器官中积累。因此,有关 AGPase 的研究主要集中在大麦、小麦、玉米等谷类作物和马铃薯等块茎类植物中,在果实中研究极少。1998 年 Kim 等首次从西瓜中分离出 AGPase 的 cDNA 克隆,并用 Northern 杂交法证明 AGPase 大亚基基因 *wml*1 表达具果实特异性[2]。刘敬梅等用 Un-even PCR 的方法分离到 *wml*1 基因 5′端启动子序列(命名为 WSP 启动子),并用瞬时表达的方法初步证实了该启动子能指导 GUS 基因在西瓜果实中特异性表达[5],为西瓜果实发育机理及在果实中定向表达外源基因的研究奠定了基础。本文在此基础上对该启动子在果实特异性表达调控中的转录调控机制作了进一步的研究,并确定了几个功能区域。

1 材料与方法

1.1 材料

京欣一号西瓜花及不同发育期果实:授粉后 5d、10d、20d,采自北京蔬菜研究中心温室,叶、茎、卷须采自组培苗。

1.2 方法

1.2.1 WSP 启动子的获得及序列分析:*wml*1 的 5′端上游长为 1864bp 的序列分离及序列分析见参考文献[5]。目的片段克隆到 pGEM-T 载体中,重组克隆命名为 pSPA。

1.2.2 亚克隆载体的构建:质粒 pSPA 用 *Hin*d Ⅲ、*Hinc* Ⅱ不完全酶切释放出 1573bp,用 *Hinc* Ⅱ、*Eco*R Ⅰ完全酶切,释放出 795bp 片段,2 个片段分别与 *Hin*d Ⅲ和 *Sma* Ⅰ、*Eco*R Ⅰ和 *Sma* Ⅰ双酶切的 pBluescript SK(-)载体连接,转化大肠杆菌 DH5α 菌株,重组质粒分别命名为 pBSPA-16、pBSPA-8。pBSPA-16 经 B*am*H Ⅰ和 *Sna*B Ⅰ、*Bam*H Ⅰ和 *Eco*R Ⅴ双酶切,回收 1201bp、898bp 片段,分别与经 *Bam*H Ⅰ和 *Sma* Ⅰ双酶切的 pBluescript SK(-)载体连接,获得重组质粒命名为 pBSPA-12、pBSPA-9。

1.2.3 瞬间表达载体的构建:重组质粒 pBSPA-16、pBSPA-12、pBSPA-9、pBSPA-8 经 *Hin*d Ⅲ和 *Bam*H Ⅰ酶切,回收相应的启动子片段后,与经 *Hin*d Ⅲ和 *Bgl* Ⅱ双酶切、切除了 CaMV 35S 启动子的植物表达载体 pBI426 连接,转化大肠杆菌 DH5α 菌株,筛选出阳性克隆,分别命名为 pISPA-16、pISPA-12、pISPA-9、pISPA-7。

1.2.4 轰击材料的处理:将新开的花及授粉后 5d、10d 和 20d 的西瓜果实在自来水中冲洗 30min 后,用 10% 的 NaCl 表

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面灭菌 10min，用灭菌蒸馏水冲洗数次；将西瓜果实纵剖开，切成薄片，与无菌培养的再生苗叶片、茎、卷须一同铺满倒有 MS0 培养基(MS + 3% 蔗糖 + 0.8% 琼脂)的 10cm × 10cm 的平皿。

1.2.5 微弹载体的制备：挑取含瞬间表达载体的菌落，在 50 mL 含有氨苄青霉素的 LB 液体培养基中培养，提取、纯化质粒，最终质粒溶解在 TE 缓冲液中，终浓度为 1 μg/μL。于旋涡振荡器充分振荡制备成金粉悬液，取 10μL，置于灭菌的 Eppendorf 管中，加入 1μL DNA 溶液，混匀。在涡旋振荡状态下缓慢加入 10μL 的 2.5 mol/L $CaCl_2$ 溶液(高压灭菌)，混匀。在同样状态下缓慢加入 4μL 0.1mol/L 亚精胺溶液(抽滤灭菌)，振荡约 3min，静置 10min。10 000r/min 离心 5s，尽可能去除上清液。将金粉悬浮于 50μL 无水乙醇中，充分振荡。10 000r/min 离心 5s，去上清。最终将制备好的金粉悬浮于 12μL 无水乙醇中。

1.2.6 装弹轰击：将 12μL 重悬颗粒均匀涂于微弹载体中心，超净工作台上吹干。将载有微粒子弹的载体及阻挡网和铺满西瓜外植体的平皿装入子弹发射装置中，抽真空、轰击。射击距离为 7cm，系统压力 1200psi，真空度 91432.23Pa。轰击后的西瓜材料于 26℃，光下培养 48h。

1.2.7 外植体 GUS 瞬间表达检测：GUS 检测按 Jefferson 等[6] 方法进行。

2 结 果

2.1 WSP 启动子不同长度片段瞬间表达载体的构建

图 1 为我们所构建的 4 个不同长度的 WSP 启动子片段的瞬间表达重组 DNA。根据 pSPA 克隆序列的酶切位点，得到 180bp-1752bp 之间的 *Hind*Ⅲ-*Hinc*Ⅱ 片段和 957bp-1752bp 之间的 *Eco*RⅠ-*Hinc*Ⅱ 片段，长度分别为 1573bp 和 795bp。两个 DNA 片段之后接上 GUS 基因和 Nos 终止子，构成转录融合体。由于瞬间表达载体 pBI426 只能用 *Hind*Ⅲ 和 *Bgl*Ⅱ 双酶切才能去除调控 GUS 基因表达的 CaMV 35S 启动子，我们选用含多克隆位点的 pBluescript SK(－)载体，其上有 *Hind*Ⅲ 和 *Bam*HⅠ(*Bgl*Ⅱ 同裂酶)切点，作为中间载体，将两个 DNA 片段亚克隆到 pBluescript SK(－)上，使他们两端获得了 *Hind*Ⅲ 和 *Bam*HⅠ 位点，最终按正确的方向插入到 pBI426 载体中，替代了 CaMV 35S 启动子。我们将获得的两个瞬间表达载体分别命名为 pISPA-16、pISPA-8。pBluescript SK(－)载体多克隆位点中 *Sma*Ⅰ 是个平端酶，pSPA 克隆序列的 551bp 和 854bp 处 *Sna*BⅠ 和 *Eco*RⅤ 也是平端酶。因此利用获得多克隆位点后的重组质粒 pBSPA-16 经 *Bam*HⅠ 和 *Sna*BⅠ、*Bam*HⅠ 和 *Eco*RⅤ 双酶切，回收 551bp-1752bp、854bp-1752bp，长度分别为 1201bp 和 898 bp 的启动子片段，与经 *Bam*HⅠ 和 *Sma*Ⅰ 双酶切的 pBluescript SK(－)载体连接，使两个片段两端也获得了 *Hind*Ⅲ 和 *Bam*HⅠ 位点，最终按正确的方向插入到 pBI426 载体中，获得的两个瞬间表达载体命名为 pISPA-12、pISPA-9。对获得的 4 个瞬间表达载体质粒(图 1)进行了限制酶鉴定，*Hind*Ⅲ + *Bam*HⅠ 双酶切后，分别释放出 0.8、0.9、1.2 和 1.6kb DNA 片段。

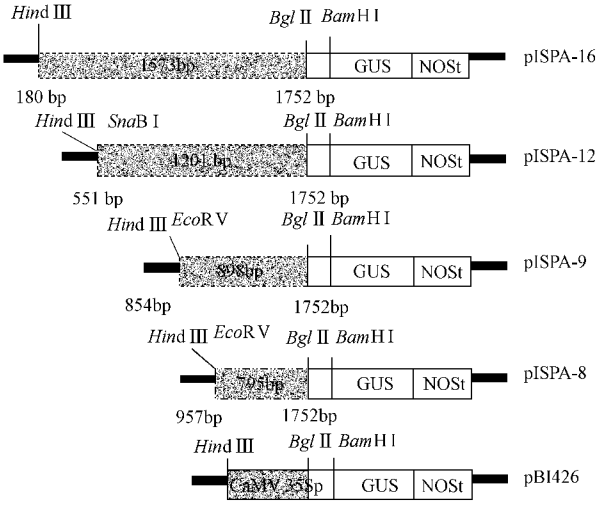

图 1 瞬间表达载体中所含的启动子区段

Fig. 1 Promoter region in transient expression vector

2.2 GUS 基因瞬间表达检测与分析

基因枪轰击后的西瓜外植体，经 GUS 染色液染色及 70% 乙醇脱色后，观察染色结果(彩版-Ⅰ)。以含有组成型启动子 CaMV 35S 启动子的 GUS 基因表达载体 pBI426 为阳性对照转化西瓜材料，在基因枪轰击后的果实、花、叶片、茎段中都出现了蓝色斑点，且表达强度都较高，表现出组成型强启动子活性。pISPA-16、pISPA-12、pISPA-9 转化的西瓜叶片、茎段、卷须等器官表面都观察不到蓝色斑点，在花中都表现出 GUS 活性。而在果实中，GUS 基因的表达情况随果实发育阶段和启动子长度而变化。pISPA-16 转化授粉 5 d、20 d 的果实表面有较强的蓝色斑点，而且 20 d 的果实中比授粉 5 d 的果实强，但授粉 10 d 的果实表面却观察不到。pISPA-12、pISPA-9 转化的果实中，只有 20 d 的果实表面有蓝色斑点，而且 GUS 基因表达的强度都比 pISPA-16 弱。表明 pISPA-16、pISPA-12、pISPA-9 所包含的启动子片段都具有启动子的功能，可以指导 GUS 基因在西瓜细胞中表达，而且，GUS 基因均只在花和果实细胞中表达，启动子具有特异性。而 pISPA-8 转化的所有西瓜外植体中都未观察到 GUS 基因表达(结果未列出)，它所包含的启动子片段不具有基本的启动子活性。

3 讨 论

果实发育过程是一个受果实特异性基因调控的过程。在果实发育过程中，许多果实特异性基因得到表达，如多聚半乳糖醛酸酶(PG)、ACC 合成酶、乙烯合成酶(EFE)和八氢番茄红素合成酶基因等[7－10] 已知功能的基因以及 2A11、E4 和 E8 等[11,12] 未知功能基因。真核生物基因的表达及其调节在很大程度上受启动子的转录调控[13]。已经发现 PG 基因 5′端调控序列的不同区域可调控报告基因在果实的不同发育期及果实的不同部位特异性表达[14]，而且具有正、负调控功能[15]。Van Haaren 等[16] 发现果实特异性基因 2A11 5′端上游序列上至少有 4 个控制果实特异性表达的区域。本实验

结果证实，瞬间表达载体 pISPA-16 所包含的 *Wml*1 基因 5′端上游 180bp-1752bp 的启动子片段能指导 GUS 在果实中特异性表达，与刘敬梅等的结果[5]相一致。

Kim 等 Northern 杂交结果发现，西瓜果实中 *Wml*1 mRNA 含量从小果期到中果期递减，然后随果实的成熟又回升[2]。这与本实验结果中 pISBA-16 轰击的授粉后 5d、20d 的果实有 GUS 表达，10d 的果实无 GUS 表达的趋势一致。这进一步证实 1573bp 的启动子包含了足以调控基因特异表达的全部信息。

898bp 的 WSP 启动子区段（pISPA-9）能启动 GUS 在果实中特异性表达，而启动子片段缩减为 795bp 时，GUS 不在任何器官中表达。这说明 854bp-958bp 之间包含了在控制 GUS 基因的表达，尤其是在果实特异性表达中必需的元件。序列分析表明，在 WSP 的 180bp-957bp 之间含有大量富含 AT 的序列。富含 AT 的序列在许多植物的基因上游是调节蛋白的结合区域，它们与蛋白之间相互作用对基因的转录起重要的调控作用[17]。其中，在 180bp-551bp 之间还含有 10bp 的反向重复序列。但 WSP 启动子序列在 GenBank 和 EMBL 中没有找到明显的同源序列。与其它果实特异性启动子 E4、E8、PG 相比，没有发现共同的保守序列存在。

在授粉 5d 的果实中，受 pISPA-16 轰击的有 GUS 表达，而受 pISPA-12 及更短启动子片段轰击的检测不到 GUS 表达，这表明 180bp-551bp 之间的片段与控制外源基因在果实发育早期表达有关。另外，在授粉 20d 的果实中，随启动子长度由 1573bp 缩短为 1201bp，GUS 的表达有减弱的趋势，说明 180bp-551bp 之间的缺失减弱了启动子的活性，该区段内可能存在促进外源基因在果实发育后期表达的顺式作用元件。基于瞬间表达结果的初步性，各启动子片段的功能有待于通过转基因植物的稳定表达来进一步证实。序列的功能及调控区域的精确定位，需要通过凝胶凝滞电泳进一步验证。

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Primary Targeting of Functional Regions Involved in Transcriptional Regulation on Watermelon Fruit-specific Promoter WSP

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Abstract Fruit ripening is associated with a number of physiological and biochemical changes. They include degradation of chlorophyll, synthesis of flavor compounds, carotenoid biosynthesis, conversion of starch to sugars, cell wall solublisation and fruit softening. These changes are brought about by the expression of specific genes. People are interested in the molecular mechanism involved in the regulation of gene transcription during fruit ripening. Many fruit-specific promoters such as PG, E4, E8, and 2A11 have been characterized and shown to direct ripening-specific expression of reporter genes. AGPase plays the key role in catalyzing the biosynthesis of starch in plants. It is a heterotetrameric enzyme with two small subunits and two large subunits, which are encoded by different genes. In higher plants, small subunits are highly conserved among plant species and expressed in all tissues. And the large subunits are present at multiple isoforms and expressed in a tissue-specific pattern. In fruits, the expression pattern of the large subunits varies with plant species. That made it important to study the transcriptional regulation of the large subunits of AGPase in different plant species. Northern-blot analysis indicates in watermelon, an isoform of the large subunits *Wml*1 expressed specifically in fruits, not in leaves. The 5′flanking region of *Wml*1, which covers 1573bp, has been isolated through the method of uneven PCR. And transient expression assay has shown that the 1573bp (named WSP) can direct fruit-specific expression of GUS gene. Our goal in this study was to scan the promoter region for main regulatory regions involved in fruit-specific expression. A chimaeric gene was constructed containing the WSP promoter, the β-glucuronidase(GUS) structural sequence as a reporter gene and the nopaline synthase polyadenylation site(NOS-ter). The plasmid pSPA was digested with *Hin*dⅢ + *Hin*cⅡ and promoter fragment of 1573bp(from 180bp to 1752bp) was cut out and cloned into *Sma* Ⅰ sites of pBluescript SK(-), to produce pBSPA-16. The same insert was then cut out with *Hin*dⅢ + *Bam*HⅠ, and ligated with transient expression vector pBI426 digested by *Hin*dⅢ + *Bgl*Ⅱ to produce pISPA-16. Three 5′-end deletions of the promoter were obtained and fused to GUS gene in plant transient expression vector pBI426: the 1201bp fragment (from 551bp to 1752bp) was generated by digestion of pBSPA-16 with *Bam*HⅠ + *Sna*BⅠ, the 898bp fragment (from 854bp to 1752bp) by *Bam*HⅠ + *Eco*RⅤ. Both fragments were ligated with pBluescript SK(-) digested by *Bam*HⅠ + *Sma* Ⅰ, to produce pBSPA-12 and pBSPA-9. The inserts were cut out with *Hin*dⅢ + *Bam*HⅠ and ligated with pBI426 digested by *Hin*dⅢ + *Bgl*Ⅱ, to produce pISPA-12 and pISPA-9. The 795bp fragment (from 957bp to 1752bp) was generated by digestion of pSPA with *Hin*cⅡ + *Eco*RⅠ, promoter fragment was cut out and cloned into *Sma* Ⅰ sites of pBluescript SK(-), to produce pBSPA-8. The same insert were cut out with *Hin*dⅢ + *Bam*HⅠ, and ligated with transient expression vector pBI426 digested by *Hin*dⅢ + *Bgl*Ⅱ. The 1573bp fragment and three 5′-end deletions were delivered into watermelon leaf, stem, flower and fruit of different development stages (5,10,20 days after pollination) via particle bombardment using a biolistic PDS-1000/He particle gun. Bombardment parameters were as follows: a helium pressure of 1 200psi, vacuum of 91432.23Pa, 7cm between the stopping screen and the plate. Histochemical assay were done on all the tissues bombarded after incubation for 2 days. The 1573bp fragment had the strongest promoter activity, and can induce GUS expression in fruits of 5 and 20 days after anthesis and flowers, but not in fruits of 10 days after anthesis, leaves and stems. Fragments of 1201bp and 898bp can induce GUS expression only in fruits of 20 days after anthesis, and with lower expression levels than 1573bp. Fragment of 795bp was not able to direct GUS expression in any of the tissues bombarded (data not shown). It can be concluded that of the 1573bp, 1201bp, 898bp *Wml*1 5′flanking regions include the necessary information directing fruit-specific expression. Deletion from 180bp to 551bp doesn't affect the fruit-specificity of the promoter, but lowered the expression level. There may be some cis-acting elements located in this region, which can enhance external gene expression in later stages of fruit development. Deletion from 854bp and 958bp led to loss of GUS expression. This region includes the necessary information needed for gene expression as well as the regulatory elements for fruit-specific transcription.

Key words watermelon, fruit-specific, promoter, transient expression

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