A/T-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants

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Received 21 November 1997; accepted in revised form 22 February 1998

Key words: enhancer, patatin, plastocyanin, potato, tobacco

Abstract

The role of an A/T-rich positive regulatory region (P268, –444 to –177 from the translation start site) of the pea plastocyanin gene (*PetE*) promoter has been investigated in transgenic plants containing chimeric promoters fused to the β -glucuronidase (GUS) reporter gene. This region enhanced GUS expression in leaves of transgenic tobacco plants when fused in either orientation to a minimal pea *PetE* promoter (–176 to +4) and in roots when fused in either orientation upstream or downstream of a minimal cauliflower mosaic virus 35S promoter (–90 to +5). The region was also able to enhance GUS expression in microtubers of transgenic potato plants when placed in either orientation upstream of a minimal class I patatin promoter (–332 to +14). Dissection of P268 revealed that *cis* elements responsible for enhancing GUS expression from the minimal *PetE* promoter were distributed throughout P268. Multiple copies of a 31 bp A/T-rich sequence from within P268 and of a 26 bp random A/T sequence were able to enhance GUS expression from the minimal *PetE* promoter, indicating that A/T-rich sequences are able to act as quantitative, non-tissue-specific enhancer elements in higher plants.

Abbreviations: CaMV, cauliflower mosaic virus; GUS, β -glucuronidase; HMG, high-mobility group; MAR, matrix-associated region; MU, methylumbelliferone; SAR, scaffold-associated region.

Introduction

The pea plastocyanin gene (*PetE*) is a single-copy nuclear gene encoding a 10 kDa copper protein which participates in photosynthetic electron transfer between cytochrome *f* and the primary donor P700 of photosystem I [32]. The *PetE* gene is expressed only in photosynthetic tissues and its expression is induced by light [7, 33, 36, 42, 47, 60]. The pea *PetE* promoter is one of the strongest photosynthesis gene promoters yet described, resulting in expression of the GUS reporter gene at levels 6-fold higher than a tobacco *RbcS* promoter and 10-fold higher than the cauliflower mosaic virus (CaMV) 35S promoter in leaves of transgenic tobacco plants [47]. Promoter-deletion analysis in transgenic tobacco plants indicated that the high-

level expression was due to the presence of a positive regulatory region from –444 to –177 upstream of the translation start site [47]. Deletion of this highly A/T-rich region (P268) resulted in markedly decreased expression in all chloroplast-containing tissues of the plants, indicating that the region –176 to +4 (relative to the translation start) was able to direct tissue-specific expression [47]. Gel retardation and DNase I footprinting experiments have shown that high mobility group proteins, HMG-1 and HMG-I/Y, bind to numerous A/T-rich DNA tracts within P268 [47, 61].

Many plant nuclear genes contain enhancer elements responsible for regulated gene expression located upstream of the transcription start sequences. Enhancer elements were first described from animal viruses [3, 41] and were able to stimulate gene ex-

pression when placed in either orientation upstream or downstream of the gene. The enhancers were able to act over considerable distances in many different tissues due to the presence of multiple *cis* elements which acted combinatorially [22]. The best characterised plant enhancer is probably that of the CaMV 35S promoter which contains multiple *cis* elements that can function either independently or synergistically to activate expression in a developmentally regulated and tissue-specific manner [4]. Many plant genes have been shown to contain upstream sequences which are able to enhance expression when placed in either orientation upstream from a minimal promoter [14, 16, 17, 18, 53, 55, 57]. These enhancer sequences often direct tissue-specific and/or regulated expression, for example by light, growth regulators or drought, from multiple *cis* elements [19, 55, 56, 63], although quantitative enhancement has been observed with some A/T-rich enhancer elements [12, 15, 49]. In general, plant enhancers direct much lower levels of expression when placed downstream, rather than upstream, of the gene [14, 29, 55]; this may be an effect of the distance of the enhancer elements from the promoter.

The aim of the experiments described in this paper was to investigate the mode of action of the positive regulatory region (P268) of the pea *PetE* promoter. In particular, we wished to examine whether P268 was able to act as an enhancer element and, if so, whether it contained any information for tissue-specific or regulated gene expression. The properties of P268, and subfragments therefrom, were investigated in transgenic tobacco and potato plants, and these experiments indicate that A/T-rich regions of DNA are able to act as quantitative, non-tissue-specific enhancers of gene expression from several plant promoters. Multiple copies of a random 26 bp A/T oligonucleotide were also able to enhance gene expression from the minimal *PetE* promoter.

Materials and methods

Generation of chimeric gene constructs

All plasmids were constructed with standard recombinant DNA techniques essentially according to Sambrook *et al.* [50] and confirmed by sequencing.

PetE promoter fusions

Several constructs containing the minimal *PetE* promoter (−176 to +4 from the translation start site) fused to the GUS reporter gene and *nos* terminator were produced from pKHh12, containing the −444 to +4 *PetE* promoter fragment fused to the GUS reporter gene and *nos* terminator in pBI101.2 [47]. In order to provide suitable restriction sites to remove the −444 to −177 promoter fragment, the −444 to +4 *PetE*-GUS-*nos* region was removed from pKHh12 as a 2.5 kb *HindIII*-*EcoRI* fragment and inserted into *HindIII* and *EcoRI*-cut pBCSK+ (Stratagene) to give pJSS1. A 2.5 kb *Sall*-*EcoRI* fragment was then cleaved from pJSS1 and inserted into *Sall*- and *EcoRI*-cut pUC19 to give pJSS3, from which the region −444 to −177 was removed by digestion with *Clal* and *DraIII*. The ends of the plasmid were made flush with T4 DNA polymerase and religated by T4 DNA ligase to give pJSS4, containing the −176 to +4 *PetE*-GUS-*nos* region in pUC19. The insert was removed as a 2.3 kb *Sall*-*EcoRI* fragment and inserted into pBIN19 [6] to give pJSS22. The insert was also removed from pJSS4 as a 2.3 kb *PstI*-*EcoRI* fragment and inserted into *PstI* and *EcoRI*-cut pBCSK+ to give pJSS53. The 2.3 kb fragment was then cut out with *XbaI* and *EcoRI* and inserted into *XbaI* and *EcoRI*-cut pUC19 to give pJSS60, or with *XbaI* and *HindIII* and inserted into *XbaI* and *HindIII*-cut pUC19 to give pJSS85.

P268 was isolated from pPC-DV4, a derivative of pPC-PC5 [47], obtained by digestion of pPC-PC5 with *EcoRV* and *DraIII* and religation after removing overhanging ends with T4 DNA polymerase. P268 was removed from pPC-DV4 (−992 to −180 in pUBS) as a *HindIII* fragment containing an additional 4 bp of polylinker at the 3' end. The *HindIII* fragment was inserted in both orientations into the *HindIII* site of pBIN19 to give pJSS15 and pJSS16. The orientation of the insert was determined by diagnostic restriction digests with *HinfI*. The 2.3 kb *Sall*-*EcoRI* fragment containing the −176 to +4 *PetE*-GUS-*nos* region was isolated from pJSS4 and was ligated into the *Sall* and *EcoRI* sites of pJSS15 and pJSS16 to obtain pJSS20 and pJSS21. These constructs contain P268 in both orientations upstream of the −176 to +4 *PetE* minimal promoter.

Subfragments of P268 were obtained from pPC-DV4 by digestion with *HindIII* and *SpeI* generating 56 bp *HindIII*-*SpeI* (−444 to −389), 105 bp *SpeI* (−388 to −284) and 161 bp *HindIII*-*SpeI* (−444 to −284) fragments, or from pPC-H268 (268 bp *HindIII* fragment from pPC-DV4 in pUBS) by digestion with

SpeI and *SalI* generating 116 bp *SpeI-SalI* (–283 to –180, with 12 bp of polylinker sequence at 3' end) and 221 bp *SpeI-SalI* (–388 to –180, with 12 bp of polylinker sequence at 3' end) fragments. The 56 bp *HindIII-SpeI* fragment was inserted into pJSS60 at the *HindIII* and *SpeI* sites to give pJSS66. The 2.4 kb *HindIII-EcoRI* fragment containing the reporter construct was cut out of pJSS66 and inserted into the *HindIII* and *EcoRI* sites of pBIN19 give pJSS74. The 105 bp *SpeI* fragment was inserted into pJSS53 at the *SpeI* site to give pJSS54. The 2.4 kb *NotI-EcoRI* fragment containing the reporter construct was excised from pJSS54 and inserted into the *NotI* and *EcoRI* sites of pBinNot (a pBIN19 derivative containing a unique *NotI* site in the polylinker, A.R. Walker, unpublished) to give pJSS79. The 116 bp and 221 bp *SpeI-SalI* fragments were inserted into the *SpeI* and *SalI* sites of pJSS60 to give pJSS87 and pJSS102, respectively. The 2.4–2.5 kb *HindIII-EcoRI* fragments containing the reporter constructs were cut out of pJSS87 and pJSS102 and inserted into pBIN19 at its *HindIII* and *EcoRI* sites to give pJSS90 and pJSS111, respectively. The 161 bp *HindIII-SpeI* fragment was inserted into the *HindIII* and *SpeI* sites of pBCSK+ give pJSS103. The 182 bp *XbaI-SalI* fragment was cut out of pJSS103 and inserted into the *XbaI* and *SalI* sites of pJSS85 to give pJSS104, from which a 2.4 kb *Asp718I-EcoRI* fragment containing the reporter construct was inserted into pBinNot to give pJSS105.

The double-stranded 31 bp oligonucleotide (–289 to –259 bp) 5'-AATATACTAGTATTATTTACTAAA-AAAATC-3' with *BamHI* overhangs added at the 5' ends was inserted as single and triple copies into the *BamHI* site of pBCSK+ to give pJSS108 and pJSS110, respectively. The 2.3 kb *PstI-EcoRI* fragment containing the –176 to +4 *PetE*-GUS-*nos* construct was removed from pJSS60 and inserted in pJSS108 and pJSS110 at the *PstI* and *EcoRI* sites. These reporter constructs were excised as *EcoRI-NotI* fragments and inserted into pBinNot opened at its *NotI* and *EcoRI* sites to give pJSS134 (1 copy) and pJSS133 (3 copies).

A random poly dAdT sequence was designed by coin flipping and constructed from two complementary oligonucleotides (5'-AATTATAATATAATTTTAAATTTAAAA-3') and (5'-AATTTTTTAAATTTAAAA-TTATATTAT-3') which contain *EcoRI* overhangs at the 5' ends to allow multimerisation without any intervening G/C bases. Oligonucleotides were annealed, phosphorylated and ligated before insertion into the *EcoRI* site of pIC19H [38]. Sequencing identified

plasmids pJSS136, pJSS137 and pJSS138 containing inserts of 4, 2 and 1 copies of the oligonucleotide, respectively. Inserts were excised as *HindIII-SalI* fragments and inserted in pJSS22 to give pJSS139, pJSS140 and pJSS141 containing 2, 1 and 4 copies respectively.

CaMV 35S promoter fusions

A –90 to +5 bp *CaMV* 35S promoter fragment upstream of the GUS reporter gene and *nos* terminator was amplified from pBI121.1 [26] using *pfu* DNA polymerase (Stratagene) and oligonucleotide primers (forward primer 5'-CCGGAATTCATCTCCACTGACGTAAGG-3' and reverse primer 5'-TCCCAGTTCACGACGT-3') designed to complement the *CaMV* 35S promoter at –90 and to vector sequence flanking the *nos* terminator. The amplified 2.2 kb fragment was digested with *EcoRI* and inserted into the *EcoRI* site of pBCSK+ to give pJSS6 and pJSS7 with the inserts in opposite orientations. The insert was excised from pJSS6 with *SalI* and *NotI* and inserted into the *SalI* and *NotI* sites of pBinNot to give pJSS14. P268 obtained as a *HindIII* fragment from pPC-DV4 was inserted into the *HindIII* site of pJSS6 to give pJSS8 and pJSS9 with P268 in both orientations downstream of the reporter construct, and into pJSS7 to give pJSS10 and pJSS11 with P268 in both orientations upstream of the *CaMV* 35S –90 promoter. The 2.5 kb *SalI-NotI* fragments from pJSS10 and pJSS11 were inserted into pBinNot at its *SalI* and *NotI* sites to give pJSS18 (P268 in the normal orientation) and pJSS19 (P268 in the reverse orientation). The 2.5 kb *NotI-Asp718I* fragments from pJSS8 and pJSS9 were inserted into pBinNot at its *NotI* and *Asp718I* sites to give pJSS12 (P268 in the normal orientation) and pJSS13 (P268 in the reverse orientation). pBI101.2 containing a promoterless GUS-*nos* construct [26] was used to provide negative control plants.

Patatin promoter fusions

Heterologous gene constructs were produced from pPS20A-G [62] which contains 2.5 kb of 5'-upstream sequence from the class I patatin gene PS20 [39] transcriptionally fused to the GUS reporter gene and *nos* terminator in pBin19. A construct containing the minimal –332 to +14 bp patatin promoter, GUS reporter and *nos* terminator was generated by excising a 2.5 kb *XbaI-EcoRI* fragment from pPS20A-G and inserting in to the *XbaI* and *EcoRI* sites of pBin19, to give pJSS116. Constructs containing P268 in the normal and reverse orientations upstream of the min-

imal patatin promoter were obtained by inserting the 2.5 kb *XbaI-EcoRI* fragment from pPS20A-G into the *XbaI* and *EcoRI* sites of pJSS15 and pJSS16 to give pJSS112 and pJSS114, respectively. pPS20A-G served as a positive control for potato transformation.

Plant transformation

Tobacco

Chimeric gene constructs in binary vectors were transferred into *Agrobacterium tumefaciens* strain LBA4404 by electroporation [52] and the resulting strains were used to infect leaf discs of tobacco (*Nicotiana tabacum* cv. Samsun) essentially according to Horsch *et al.* [23]. The regenerated plants were dissected from callusing leaves and maintained in sterile culture on MS media containing carbenicillin (200 µg/ml) and kanamycin (100 µg/ml) and subcultured every 2 weeks. The rooted transgenic plants obtained within 10–12 weeks were transferred to soil and acclimatised in a propagator before growth in a controlled environment room (Fisons Sanyo). The plants were allowed to flower, inflorescences were bagged to prevent cross-pollination and resulting seed was collected as the T₁ generation.

Potato

A. tumefaciens-mediated transformation methods were used to infect leaf discs of potato (*Solanum tuberosum* cv. Prairie) essentially according to Horsch *et al.* [23]. Infected discs were allowed to grow on MS medium containing 3% sucrose for 3 days and transformants were selected on media described by Blundy *et al.* [8]. Callus was induced on MS medium supplemented with benzylaminopurine (2.5 µg/ml), naphthalene acetic acid (0.1 µg/ml), GA₃ (10 µg/ml), claforan (500 µg/ml) and kanamycin (50 µg/ml). Calli were transferred to MS medium containing benzylaminopurine (2.5 µg/ml) and GA₃ (10 µg/ml) for shoot induction. Shoots were excised and cultured on liquid MS medium without hormones and subcultured every 2 weeks. Strong shoots were transferred onto solid MS medium without hormones to make them sturdy. Microtubers were induced by placing the nodal cuttings from the sturdy shoots in MS medium supplemented with 6% sucrose, kinetin (2.5 µg/ml) and ancymidol (α -cyclopropyl- α -(*p*-methoxyphenyl)-5-pyrimidine methanol, 180 µg/ml [45]). Tubers were induced after 5–6 weeks incubation in dark at 19 °C.

GUS assays

GUS enzyme analysis were performed essentially according to Jefferson *et al.* [26]. About 50 mg of plant tissue was homogenised in 300 µl GUS lysis buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% sodium laurylsarcosine, 10 mM 2-mercaptoethanol), and 10–30 µl of the homogenate was used in each assay containing 1 mM 4-methylumbelliferyl β -glucuronide. Fluorescence was measured with a Perkin Elmer luminescence spectrometer LS50 (Perkin Elmer, Norwalk, CT). The GUS-Light chemiluminescence assay system (Tropix, Bedford, MA) was used to measure GUS activity in root cell lysates because the fluorometric GUS assay was not linear with respect to time and extract concentration due to quenching of fluorescence. Absolute GUS activity was quantified using purified β -glucuronidase (Sigma G-7896) assayed by both fluorometric and chemiluminescence assays. Chemiluminescence was measured with a Luminoskan RS luminometer (Life Sciences International, Basingstoke, UK). GUS activity was expressed as pmol MU per minute per µg protein. Protein concentration was determined by the microassay procedure of Bradford [9]. *t*-tests were carried out to determine whether there were statistically significant differences between GUS activities of populations of plants transformed with different constructs.

Results

The positive regulatory region P268 behaves as an enhancer

The ability of P268 to enhance the expression of the GUS reporter gene in transgenic tobacco plants was examined. Chimeric promoter constructs containing P268 in either orientation fused to a minimal pea *PetE* promoter (–176 to +4) were generated (Figure 1). These chimeric genes were transferred to tobacco by *Agrobacterium*-mediated transformation. The results obtained from the measurement of GUS activity in leaves of tobacco plants in culture are summarised in Figure 1. No GUS activity was detected in plants containing the promoterless pBI101.2 construct, and only low activity was obtained from plants containing the minimal *PetE* promoter construct. The presence of P268 in normal and reverse orientations enhanced expression from the minimal *PetE* promoter by 12- and 13-fold respectively. This indicated that P268 was able

Figure 1. P268 enhances expression of the GUS reporter gene from a minimal *PetE* promoter in transgenic tobacco. The reporter gene constructs are shown diagrammatically on the left, with the name of the plasmid used for transformation. The arrows indicate the orientation of P268. The GUS activity of leaf extracts is shown on the right. GUS activity was measured fluorometrically with methylumbelliferyl glucuronide as substrate. GUS activity is given as the mean $\pm$ standard error. The numbers of plants assayed were: pBI101.2, $n = 6$; pJSS22, $n = 6$; pJSS20, $n = 10$; pJSS21, $n = 8$.

to act as an enhancer in leaves of transgenic tobacco plants. GUS activity was not detected in root tissue from transgenic plants containing any of the constructs (data not shown), as expected if tissue-specific expression was directed solely by the minimal *PetE* promoter [47].

P268 enhances expression from a CaMV 35S –90 promoter in root tissue

To determine whether P268 was able to enhance expression in non-photosynthetic tissues, chimeric genes containing P268 fused in all possible orientations upstream and downstream of a CaMV 35S –90 promoter (–90 to +5) were transferred to tobacco (Figure 2). The CaMV 35S –90 promoter directs GUS expression mainly in roots with much lower, if any, expression in leaf tissue [5]. The expression from these chimeric promoter constructs was analysed in root and leaf tissue of transgenic tobacco plants using a chemiluminescence assay for GUS activity (see Materials and methods). The fluorometric GUS assay was not used because it was not linear with respect to time or extract concentration due to quenching of fluorescence by root extracts. The data shown in Figure 2 reveal that all four constructs containing P268 were able to enhance expression from the CaMV 35S –90 promoter in root tissue. GUS activity of root tissue from transgenic plants containing P268 in the normal and reverse orientations upstream of the CaMV 35S –90 promoter was 6- and 9-fold higher, respectively, than the activity from the minimal promoter alone. GUS activity of root tissue from transgenic plants containing P268 fused in

either orientation downstream of the CaMV 35S –90 promoter was enhanced ca. 2-fold.

Although the CaMV 35S –90 promoter has been reported to direct only low levels of expression in leaf tissue [5], all transgenic plants were analysed for the presence of GUS activity in leaves (Figure 2). Low levels of GUS activity were detected in the leaves of all plants, except those containing the promoterless pBI101.2 construct. In plants containing the CaMV 35S –90 promoter the leaf GUS activity (0.3 ± 0.1 pmol min^{–1} μg^{–1}) was about 16% of the root activity (2.1 ± 0.5 pmol min^{–1} μg^{–1}). GUS expression in leaves was enhanced ca. 2- and 3-fold when P268 was placed upstream of the CaMV 35S –90 promoter in the normal and reverse orientations, respectively (Figure 2). However, there were no discernable effects on GUS activity in leaves when P268 was placed downstream of the GUS coding region and the *nos* terminator (Figure 2).

These results demonstrate that the presence of P268 results in enhanced expression from the CaMV 35S –90 promoter in root tissue, and to a lesser extent in leaf tissue. However, as shown in Figure 1, P268 enhanced expression in leaves but not in roots when fused to the minimal *PetE* promoter. This suggests that P268 itself does not contain any information for tissue-specific expression and is behaving solely as a quantitative enhancer.

P268 enhances expression from a minimal patatin promoter in potato microtubers

The enhancer activity of P268 was further examined in transgenic potato plants. Chimeric gene constructs

Figure 2. P268 enhances expression of the GUS reporter gene from a CaMV 35S –90 promoter in transgenic tobacco. The reporter gene constructs are shown diagrammatically on the left, with the name of the plasmid used for transformation. The arrows indicate the orientation of P268. The GUS activities of root (black bars) and shoot (dotted bars) extracts are shown on the right. GUS activity was measured luminometrically with the GUS-Light assay system. GUS activity is given as the mean $\pm$ standard error. The numbers of plants assayed were: pBI101.2, $n = 6$; pJSS17, $n = 10$; pJSS12, $n = 9$; pJSS13, $n = 11$; pJSS18, $n = 9$; pJSS19, $n = 10$.

with P268 fused in both orientations upstream of a minimal –332 to +14 promoter from a patatin class I gene (PS20) [39] were generated (Figure 3). The patatin class I genes are expressed at high levels in potato tubers [46] and the 2.5 kb full-length PS20 promoter is able to direct tuber-specific and sucrose-inducible expression in transgenic potatoes [8, 62]. Promoter-deletion analysis of related patatin class I genes has indicated that tuber-specific and sucrose-inducible expression is retained on deletion to about –300 bp [25, 35]. Transgenic potato plants were obtained using *Agrobacterium*-mediated transformation of leaf discs and microtubers were induced by incubation of transgenic stem segments on media supplemented with kinetin and ancymidol in the dark. Measurement of GUS activity in microtubers demonstrated the ability of P268 to enhance expression from the minimal patatin promoter to levels equal to the full-length patatin PS20 promoter. The transgenic microtubers containing P268 in the normal and reverse orientations showed a 4-fold increase in GUS activity compared to the minimal patatin promoter (Figure 3). No GUS activity was detected in the leaves of any of the transgenic potato plants containing patatin promoter constructs. These results support and confirm the earlier conclusion that P268 behaves as a non-tissue-specific enhancer, able to activate homologous and heterologous promoters in different plants and tissues (leaves, roots and tubers) with the tissue

specificity of expression determined by the minimal promoters [5, 25, 35, 47].

Cis elements conferring enhancer properties are distributed throughout P268

In order to define the *cis*-acting element(s) within the P268 enhancer region, the activity of subfragments of P268 fused to the minimal (–176 to +4) *PetE* promoter was examined (Figure 4). Two conveniently located *SpeI* sites were used to dissect P268 into five overlapping fragments of 56 bp (–444 to –389), 105 bp (–388 to –284), 104 bp (–283 to –180), 161 bp (–444 to –284) and 209 bp (–388 to –180). The chimeric genes were transferred to tobacco and GUS activity was measured in young leaves (Figure 4). All the subfragments of P268 were able to enhance expression when fused to the minimal *PetE* promoter (Figure 4). Transgenic plants containing the 56 bp fragment (pJSS74) or the 161 bp fragment (pJSS105) gave 6-fold increases in gene expression over the minimal *PetE* promoter, whereas the 209 bp fragment (pJSS111) was able to enhance expression 5-fold. Lower levels of enhancement were obtained with the 105 bp fragment (pJSS79) and the 104 bp fragment (pJSS90) which gave 4-fold and 3-fold enhancements of GUS activity, respectively (Figure 4). This suggests that *cis* elements responsible for enhancing gene expression are distributed throughout P268.

Figure 3. P268 enhances expression of the GUS reporter gene from a minimal patatin promoter in transgenic potato. The reporter gene constructs are shown diagrammatically on the left, with the name of the plasmid used for transformation. The GUS activity of extracts of microtubers is shown on the right. GUS activity was measured fluorometrically with methylumbelliferyl glucuronide as substrate. GUS activity is given as the mean $\pm$ standard error. The numbers of plants assayed were: pJSS116, $n = 12$; pPS20A-G, $n = 19$; pJSS112, $n = 23$; pJSS114, $n = 26$.

A 31 bp A/T-rich region enhances gene expression

DNase I footprinting assays performed to identify the sequences in P268 bound by nuclear proteins have demonstrated the binding of HMG proteins to A/T-rich DNA tracts throughout P268 [47, 61]. A 31 bp region (–259 to –289) of P268 which was strongly protected from DNase I digestion by HMG proteins and comprises highly A/T-rich (80%) DNA was selected to examine whether this fragment enhances expression from the minimal *PetE* promoter. Chimeric gene constructs containing single and triple copies of this 31 bp region were generated from synthetic oligonucleotides synthesised with additional *Bam*HI overhanging ends and fused in the normal orientation upstream of the minimal *PetE* promoter (Figure 5). These constructs were transferred to tobacco and GUS activity was measured in leaves. The presence of the 31 bp sequence enhanced GUS gene expression in comparison to the minimal *PetE* promoter (Figure 5). A single copy of the 31 bp sequence increased expression nearly 2-fold over the minimal *PetE* promoter, whereas triplication of the 31 bp region increased expression 4–5-fold when compared to the minimal *PetE* promoter. This suggested that enhancer activity was correlated with the number of copies of the 31 bp A/T-rich sequence.

A random A/T oligonucleotide enhances gene expression

In view of the apparent relationship between enhancer activity and A/T-rich sequences, the effect of multiple copies of a random 26 bp oligonucleotide consisting

solely of dA and dT nucleotides was examined. The sequence of the oligonucleotide was specified by coin-flipping (see Materials and methods) and chimeric gene constructs containing 1, 2 and 4 copies of the 26 bp oligonucleotide fused to a minimal pea *PetE* promoter (–176 to +4) were generated (Figure 6). These constructs were transferred to tobacco and expression of the GUS reporter gene in leaves was examined (Figure 6). The chimeric promoter construct containing a single copy of the 26 bp sequence directed GUS expression that was not significantly different to the minimal *PetE* promoter. However, the construct containing 2 copies of the 26 bp sequence gave a 4-fold increase in gene expression and the construct containing 4 copies enhanced expression 8-fold. The GUS activities of plants containing 4 copies of the 26 bp sequence (36 ± 12 pmol min^{–1} μ g^{–1}, Figure 6) were comparable to those of plants containing the 56 bp and 161 bp fragments of P268 (31 ± 8 and 33 ± 7 pmol min^{–1} μ g^{–1}, respectively; see Figure 4). These results demonstrate that A/T-rich sequences are able to enhance expression when fused to the minimal *PetE* promoter and that the enhancement is dependent on the number of copies of the A/T-rich sequence.

Discussion

The present study has shown that the A/T-rich 268 bp positive regulatory region of the pea *PetE* promoter acts as an enhancer of gene expression from both homologous and heterologous promoters in several tissues of two different transgenic plants. P268 was able

Figure 4. Enhancer elements are scattered throughout P268. The reporter gene constructs containing subfragments of P268 fused to the minimal *PetE* promoter are shown diagrammatically on the left, with the name of the plasmid used for transformation. The GUS activity of tobacco leaf extracts is shown on the right. GUS activity was measured fluorometrically with methylumbelliferyl glucuronide as substrate. GUS activity is given as the mean $\pm$ standard error. The numbers of plants assayed were: pBI101.2, $n = 6$; pJSS22, $n = 6$; pJSS74, $n = 10$; pJSS79, $n = 9$; pJSS90, $n = 7$; pJSS105, $n = 11$; pJSS111, $n = 7$.

to enhance expression of the GUS reporter gene when placed in either orientation upstream of minimal *PetE*, CaMV 35S -90 and patatin promoters. It was also able to enhance expression, although not to such a great extent, when placed downstream of the CaMV 35S -90 promoter. This indicates that P268 has the properties of a classical enhancer element.

A major difference between the pea *PetE* enhancer and those from many other plant genes is the apparent absence of any elements directing tissue-specific or other regulated expression. The *PetE* enhancer appears to act as a purely quantitative element. The tissue-specific expression detected in the transgenic tobacco and potato plants was directed by the minimal *PetE*, CaMV 35S -90 and patatin promoters. P268 enhanced expression from the *PetE* promoter in leaves, but not in roots, whereas it enhanced expression from the CaMV 35S -90 promoter in roots, with much lower activity in leaves. Expression from the minimal patatin promoter was detected only in tuber tissue and not in leaves or roots. These are the patterns of expression directed by each of these minimal promoters [5, 25, 35, 47].

Cis elements showing enhancer activity are distributed throughout the A/T-rich *PetE* enhancer region (see Figure 4), and a 31 bp highly A/T-rich (90% A/T) region was able to act as an enhancer element in a copy-number-dependent manner (Figure 5). This sug-

gested there may be a relationship between A/T-rich regions and enhancer activity. The enhancer activity shown by multiple copies of a 26 bp random oligonucleotide consisting solely of A/T bp (Figure 6) suggests that A/T-richness alone may be sufficient for enhancer activity.

A/T-rich elements have been shown to be important for quantitative expression of many plant genes [12, 13, 15, 27, 31, 43, 49, 58, 64]. However, different A/T-rich sequences have been reported to show positive [12, 15, 27, 43, 49, 58, 64] or negative [13, 28] regulatory features. A/T-rich regions from a β -phaseolin gene promoter [12], the soybean *lbc3* gene promoter [34] and a soybean heat-shock protein gene [13] have been shown to enhance expression from truncated heterologous promoters, and therefore appear to have similar properties to the pea *PetE* enhancer. It has previously been suggested that the β -phaseolin A/T-rich sequence might act as a general positive regulatory element enhancing the activity of a proximal region primarily responsible for tissue-specific expression [12]. Our results with the *PetE* A/T-rich region in transgenic tobacco and potato plants support the view that A/T-rich sequences act as general positive regulatory elements. Experimental evidence for A/T-rich sequences showing negative or neutral effects on gene expression has been gained only by deletion analysis [12, 28, 64] which may not

Figure 5. A 31 bp A/T-rich region enhances expression of the GUS reporter gene from a minimal *PetE* promoter in transgenic tobacco. The reporter gene constructs are shown diagrammatically on the left, with the name of the plasmid used for transformation. pJSS134 contains a single copy of the 31 bp sequence; pJSS133 contains 3 copies. The GUS activity of leaf extracts is shown on the right. GUS activity was measured fluorometrically with methylumbelliferyl glucuronide as substrate. GUS activity is given as the mean $\pm$ standard error. The numbers of plants assayed were: pBI101.2, $n = 6$; pJSS22, $n = 6$; pJSS134, $n = 10$; pJSS133, $n = 12$.

Figure 6. A random A/T oligonucleotide enhances expression of the GUS reporter gene from a minimal *PetE* promoter in transgenic tobacco. The reporter gene constructs are shown diagrammatically on the left, with the name of the plasmid used for transformation. pJSS140 contains a single copy of the 26 bp A/T sequence, pJSS139 contains 2 copies and pJSS141 contains 4 copies. The GUS activity of leaf extracts is shown on the right. GUS activity was measured fluorometrically with methylumbelliferyl glucuronide as substrate. GUS activity is given as the mean $\pm$ standard error. The numbers of plants assayed were pBI101.2, $n = 6$; pJSS22, $n = 6$; pJSS140, $n = 6$; pJSS139, $n = 10$; pJSS141, $n = 5$.

have been incisive enough to separate specific negative elements from otherwise A/T-rich sequences.

In several studies these A/T-rich sequences have been shown to be binding sites for HMG proteins. Both HMG-1 and HMG-I/Y have been shown to bind to P268, although only HMG-I/Y appears to bind specifically to A/T sequences [48, 61]. Similar HMG protein binding has been observed with A/T-rich elements in several plant genes [44], including the soybean *lbc3* gene [24] and a soybean heat-shock protein gene [15]. The rice HMG-I/Y protein binds to an A/T-rich positive regulatory region in the oat phytochrome A3 gene promoter, implicating the HMG-I/Y protein in transcriptional regulation [43]. Studies on HMG

protein binding to the A/T-rich region P31 in the *PetE* enhancer have shown that HMG-1 enhances HMG-I/Y binding, suggesting that an HMG-I/Y-DNA complex is likely to form *in vivo* (C.I. Webster and J.C. Gray, unpublished results). Pea HMG-I/Y binds to sequences containing 5 bp or more A/T, whereas HMG-1 appears to recognise deformed or deformable DNA without any defined sequence specificity [61]. High-affinity binding sites for HMG-I/Y proteins have been defined as two or more A/T-tracts of 5 or more A/T bp separated by 6–8 bp [37]. A strong positive correlation is observed between the GUS activity of leaf extracts and the number of high-affinity binding sites for HMG-I/Y proteins (as defined by Maher and Nathans

Figure 7. Correlation of GUS activity with the number of high-affinity HMG-I/Y-binding sites. The numbers of high-affinity HMG-I/Y-binding sites in enhancer regions were estimated by inspection of the nucleotide sequences. A high-affinity site is defined as two A/T tracts of 5 bp or more A/T separated by 6–8 bp [37]. GUS activities (mean $\pm$ standard error) in leaf extracts of transgenic tobacco containing enhancer regions upstream of the minimal *PetE* promoter were obtained from Figures 1, 4, 5 and 6.

[37]) in the enhancer elements fused to the minimal *PetE* promoter (Figure 7). This suggests a functional role for HMG-I/Y proteins in enhancing gene expression and indicates that multiple A/T-rich, high-affinity HMG-I/Y binding sites act additively rather than multiplicatively. The precise role of the HMG-I/Y proteins is not clear but HMG proteins have been described as 'architectural' proteins modelling the chromatin structure in the vicinity of genes [20]. HMG-I/Y has been shown to compete with histone H1 for binding to A/T-rich sequences at scaffold-associated regions (SARs) of DNA in animal cells [65]. This is proposed to alter the local chromatin structure and influence expression from nearby promoters.

A link between HMG-I/Y binding at A/T-rich sequences and SARs may help to explain the enhancer activity of P268 of the pea *PetE* promoter. We have recently shown that HMG-I/Y stimulates SAR binding to pea nuclear scaffold preparations and that P268 has weak scaffold-binding activity (L.A. Watson, D. Hatton, C.I. Webster and J.C. Gray, unpublished). Several SARs (or MARs, matrix-associated regions) have been characterised from plants [2, 10, 21, 51, 54, 59] and they all appear to be highly A/T-rich regions of DNA [11]. Some of these SARs have been shown to

have the property of decreasing position-effect variation in transgenic plants presumably by insulating reporter genes from flanking chromatin structure [10, 40]. However other SARs have been shown to enhance the activity of adjacent reporter gene constructs [1, 51, 59]. This property of these SARs appears to be similar to the enhancer activity described for the positive regulatory region of the pea *PetE* gene. This may suggest that the pea *PetE* enhancer, and possibly other A/T-rich enhancer elements, acts by remodelling local chromatin structure from SARs, thus influencing expression from adjacent promoters.

Acknowledgements

We would like to thank Dr Mike Burrell and Margaret Blundy of Advanced Technologies (Cambridge) Ltd. for help with potato transformation, Dr Peter White for help with chemiluminescence-based GUS assays, and Dr Len Packman for oligonucleotide synthesis. J.S.S. was supported by Cambridge Commonwealth Trust and Cambridge Nehru Scholarships. This work was supported by a Research Grant from the Biotechnology and Biological Sciences Research Council.

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