The 22 bp W1 element in the pea lectin promoter is necessary and, as a multimer, sufficient for high gene expression in tobacco seeds

Pater, B.S. de; Pham, K.; Klitsie, I.; Kijne, J.W.

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Sylvia de Pater*, Khanh Pham, Inge Klitsie and Jan Kijne
Center for Phytotechnology, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, Netherlands (author for correspondence)*

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Abstract

The pea lectin (*Psl*) gene encodes an abundant seed protein. Its seed-specific expression pattern is conserved in transgenic tobacco plants. Progressive 5′ promoter deletions resulted in a gradual decrease of transcriptional activity in tobacco seed. A fragment of 115 bp still conferred seed-specific expression albeit at a low level. This fragment contains a 22 bp element (W1), which has been demonstrated to be important for seed-specific expression when coupled as a trimer to a heterologous TATA box (de Pater *et al.*, Plant Cell 5: 877–886, 1993). Here we show that deletion of W1 in the natural promoter context resulted in a strongly decreased level of gene expression. A 4 bp mutation of W1 reduced the expression of truncated derivatives of the *Psl* promoter. A single copy of W1 coupled to the TATA box of the CaMV 35S promoter directed low gene expression in seeds and leaves. Multimerization enhanced the expression in seeds up to 100-fold, to levels found with the *Psl* promoter, whereas the expression level in leaves remained low. These results demonstrate that the W1 element is an essential control element in the *Psl* promoter. When taken out of its natural context and multimerized, it is sufficient for high expression in seeds.

Introduction

In the present study, promoter sequences required for seed-specific gene expression have been analysed, using the pea lectin (*Psl*) gene as a model. PSL is an abundant seed protein, that is encoded by a single-copy gene [9, 12, 16]. The mRNA abundance in roots and leaves of three-week-old seedlings is more than three orders of magnitude less than in seeds [2].

To unravel the molecular basis of seed-specific gene expression, research has been focused on the identification of *cis*-acting promoter elements and *trans*-acting protein factors. Several DNA-binding proteins of the bZIP class containing a basic domain and leucine zipper were found to play a role in seed-specific gene expression. A well-studied bZIP protein that is involved in expression of endosperm-specific genes is Opaque-2 (O2) of maize [27]. Mutations in either O2 [39, 40] or its binding site in zein promoters [39] resulted in severely reduced transcriptional activation. O2 is specifically expressed in endosperm [8], which provides a mechanism for the endosperm-specific expression of the genes that are activated by O2.

Several other bZIP *trans*-acting factors have been cloned that are thought to play a role in seed-specific gene expression. The rice transcriptional activator RITA-1 is specifically expressed in aleurone and endosperm tissues during seed maturation, and may regulate (so far unidentified) genes in developing rice seeds [14]. Another bZIP protein, EmBP-1 [10], binds to an abscisic acid (ABA)-responsive element (ABRE) present in the promoter of the wheat *Em* gene. The *Em* gene is expressed during seed maturation in response to elevated levels of ABA [23]. ABA has been widely implicated as the key hormone regulating seed maturation [35]. ABREs from several ABA-responsive genes were shown to be sufficient to confer ABA

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X66368 (*Psl*).

responsiveness or seed specificity onto heterologous promoters [24, 32, 33, 36]. A synthetic sequence has been described that also functions as a seed-specific and ABA-inducible element [19], but it is not known whether this element occurs in any plant promoter.

In addition to ABA, the *trans*-activator VP1 is required for induction of the maize *Em* homologue during embryo development [26]. Both ABA and VP1 are also involved in the induction of the maize *C1* gene [11]. C1 encodes a *myb* homologue regulating anthocyanin biosynthesis in seed tissues. ABA induction and VP1 activation of *C1* is mediated by a promoter region containing a so-called Sph element or RY repeat [11]. The RY repeat is required for the function of several other seed-specific promoters [1, 6, 7, 20]. A 6 bp deletion in the RY repeat of a legumin gene of *Vicia faba* drastically reduced expression in seed [1]. RY repeats were also found to influence the expression of genes encoding soybean β-conglycinin [7], bean glycinin [20], and a non-storage seed protein of *V. faba* [6].

None of the promoter elements mentioned above other than the ABREs have been shown to function as seed-specific enhancers in the absence of other sequence elements. By a gain-of-function approach we have shown previously that a 22 bp sequence derived from the *Psl* promoter confers seed-specific gene expression when coupled to a heterologous TATA-box [31]. This sequence, W1, contains a high-affinity binding site for certain bZIP proteins, including O2 [30]. Here we show in a loss-of-function approach that W1 is necessary for the function of the *Psl* promoter. Moreover, multimers of this sequence conferred a high level of expression similar to the expression level conferred by the intact *Psl* promoter.

Materials and methods

Gene construction and plant transformation

The pea lectin gene was isolated by screening a genomic library of pea (*Pisum sativum* cv. Feltham First) [9] with the PSL encoding cDNA clone pMS2 [38]. A subclone containing 2116 bp of promoter sequences was designated LEC1 (Fig. 1). 5′ deletion derivatives were generated using the following restriction enzymes: *Bgl*II (LEC2), *Dra*I (LEC3), *Mnl*I (LEC5), *Taq*I (LEC6), and *Mlu*I (LEC7). LEC1 to LEC7 were cloned in the binary vector pSDM300 [28].

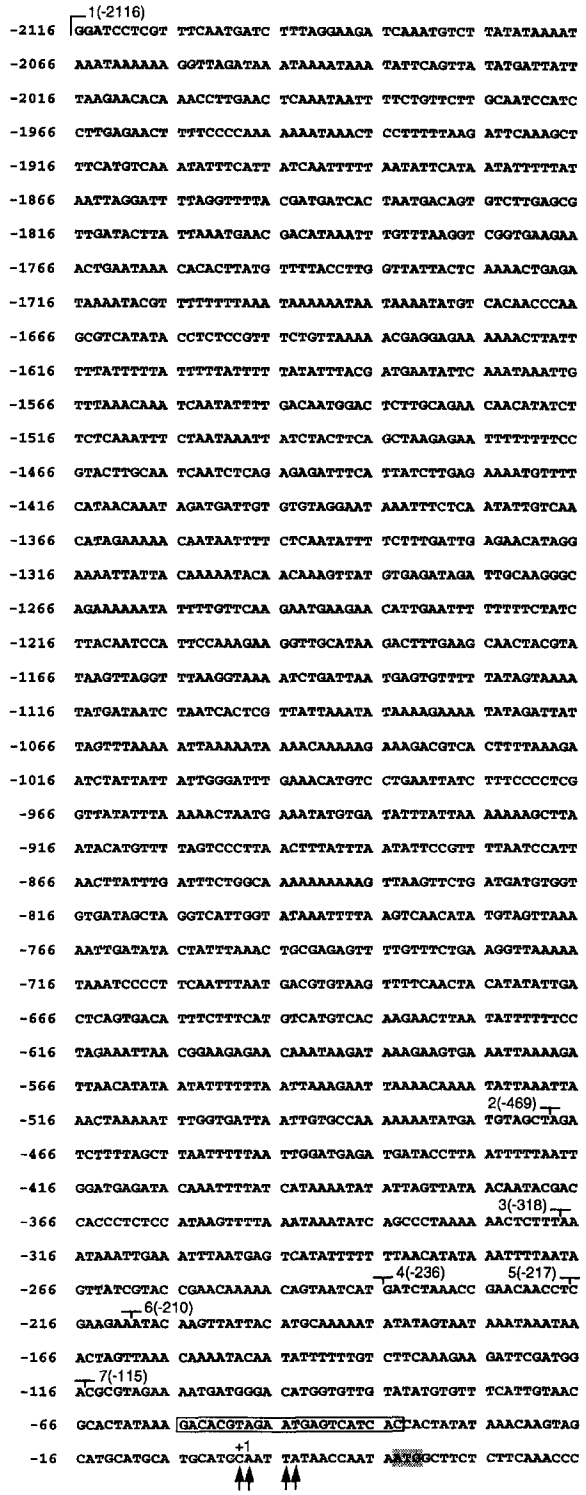

Figure 1. Nucleotide sequence of the *Psl* promoter. The positions of 5′ deletion end points are indicated. The numbers (1 to 7) of the 5′ deletions refer to the numbers of the constructs (Fig. 2). The 22 bp W1 sequence is boxed. The 5′ ends of the mRNA are indicated by arrows and the translation start codon is shaded.

Table 1. Nucleotide sequence of the W1 element and derivatives thereof present in the constructs indicated. W1 is given in upper case and flanking and mutated sequences in lower case.

Psl promoter	taaaGACACGTAGAATGAGTCATCACcact
LG2-del, LG4-del, LG7-del	taaa ..... gtcgac ..... cact[a]
LG2-mu, LG4-mu, LG7-mu	taaaGACACGTAGAATaAcTtATaAC cact
W1-GUS to 9W1-GUS	ctcgagGACACGTAGAATGAGTCATCACgtcgac

[a] Dots represent deleted nucleotides.

Psl-gusA constructs were made using the vector system of Pasquali *et al.* [29]. An *Nco*I site was introduced at the ATG start codon of the *Psl* gene. The 2116 bp *Bam*HI-*Nco*I promoter fragment or the 5′ deletion fragments *Bgl*II-*Nco*I, *Sau*3AI-*Nco*I or *Mlu*I-*Nco*I were cloned in the promoter probe plasmid GusSH [29], resulting in the constructs LG1, LG2, LG4, and LG7, respectively. Promoter fragments containing a deletion of the 22 bp W1 element or nucleotide substitutions within this element (Table 1) were produced by PCR. All *Psl-gusA* constructs were cloned as *Xba*I-*Xho*I fragments in the binary vector pMOGλCAT [29]. As a negative control the construct GusSH-47 [29] was used which contains the TATA box of the CaMV 35S promoter (–47 to +27) coupled to the *gusA*-coding region and the *RbcS*-3C terminator.

A monomer and multimers of the 22 bp W1 element (Table 1) were fused to –46GUS [31], containing the CaMV 35S promoter TATA box (–48 to +8), the *gusA*-coding region and the *RbcS*-3C terminator. As a positive control construct, an intron-containing *gusA* gene, which is under control of the CaMV 35S promoter (–418 to +2; 35SGUSINT) was used [41]. These constructs as well as –46GUS and 35SGUSINT were cloned in the binary vector pSDM300 [28]. All binary vectors were transferred to the disarmed *Agrobacterium tumefaciens* strain MOG101 [13] via triparental conjugation with *E.coli* HB101 containing pRK2013. Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) was transformed with the MOG101 derivatives by the leaf disk transformation method.

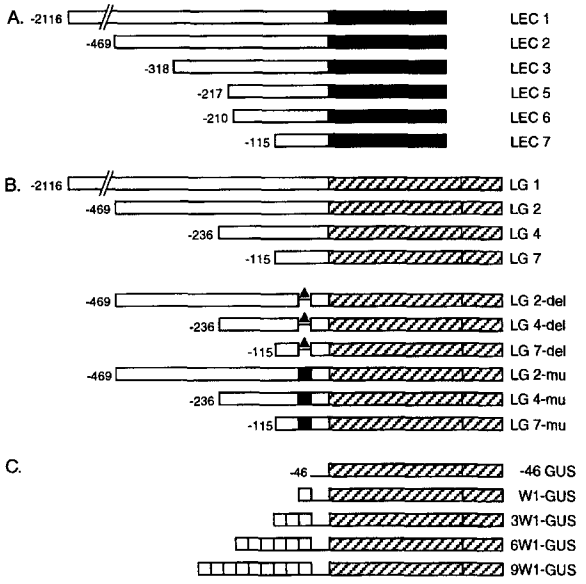

Figure 2. Structure of *Psl* promoter constructs. A. *Psl* gene and 5′ deletion derivatives. B. Chimeric *Psl* promoter-*gusA* fusions and derivatives. C. –46GUS and derivatives containing 1 to 9 copies of the W1 element. Positions of 5′ end points are indicated. The *Psl* promoter region is indicated by open bars, *Psl*-coding region and terminator by black bars, the *gusA*-coding region and the *RbcS*-3C terminator by hatched bars. Deletions and mutations in LG2, LG4 and LG7 are indicated by triangles and black squares, respectively.

ELISA

Transgenic tobacco plants containing the *Psl* gene and 5′ deletion derivatives were tested for PSL content in leaf and root tissue and mature seeds using enzyme-linked immunosorbant assays (ELISA). Protein extracts were prepared in 10 mM NaH_2PO_4/NaOH (pH 9.5); 0.15 M NaCl, and 0.2 to 2 μg of soluble protein were tested for the presence of PSL using polyclonal antibodies against native PSL [4]. Protein concentrations in extracts were determined using the BioRad protein assay dye reagent.

Fluorometric GUS assays and histochemical staining

Transgenic tobacco plants were analysed for GUS activity according to the method of Jefferson [15] using 4-methylumbelliferyl glucuronide as a substrate. The reactions were performed in microtiter well plates for 2 h (high activities) to 30 h (low activities), using 1 to 20 μg of protein. For leaf and root protein extracts *in vitro* grown plants were used, whereas for seed protein extracts, plants were grown in a greenhouse.

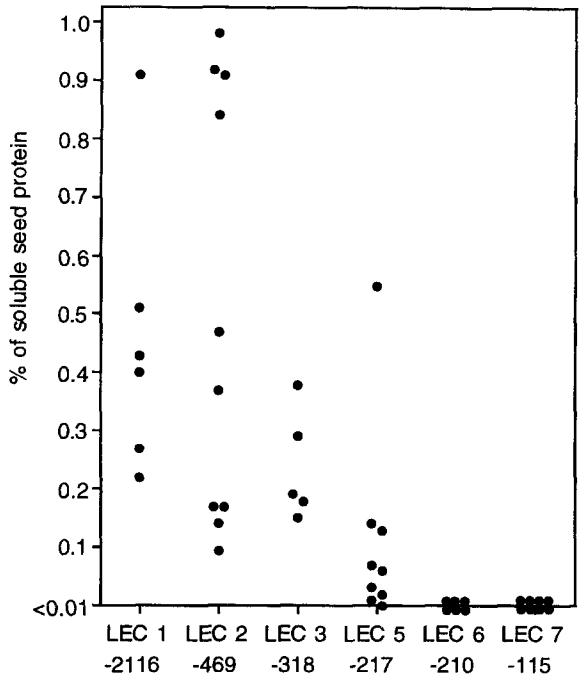

Figure 3. The *Psl* gene is expressed in transgenic tobacco seeds. PSL protein was quantitated by ELISA using anti-PSL antibodies in extracts of soluble seed protein from tobacco plants transformed with the *Psl* gene and 5′ deletion derivatives.

Product formation was quantified in a fluorometer (model LS50; Perkin-Elmer), and GUS activity was calculated by linear regression analysis of the values obtained. GUS activity is given in pmol of 4-methylumbelliferone (MU) formed in 1 min per mg of protein. All sets of data were analysed by the Wilcoxon test, and found to be different at the 5% significance level ($P<0.05$) unless stated otherwise.

Results

Seed-specific expression of the Psl *gene in transgenic tobacco plants*

To analyse its tissue-specific expression pattern, the *Psl* gene including 2116 bp of the 5′ non-coding region (LEC1; Fig. 1 and 2A) and 401 bp of the 3′ non-coding region was introduced into tobacco by leaf disc transformation. The amounts of PSL protein in leaf, root and seed of the transformed plants were determined by ELISA with anti-PSL antibodies. No PSL could be detected in leaf and root extracts (data not shown). In contrast, the amounts of PSL in seeds of individual transformants varied from 0.2% to 0.9% of total soluble protein (Fig. 3), with a mean value of about 0.5%. Thus, the *Psl* gene is expressed specifically in seeds of transgenic tobacco plants.

To determine the minimum length of the *Psl* promoter required for high expression in seed, progressive 5′ deletions were generated (Fig. 1 and 2A), and the truncated genes were introduced into tobacco. A *Psl* gene construct containing the 469 bp promoter (LEC2) still gives high PSL levels in seed of about 0.5% of total soluble protein (Fig. 3). The amount of PSL in seed of LEC3 plants (318 bp promoter) was lower (0.24%) than the amounts in seed of LEC1 and LEC2 plants, but this difference was not significant. Deletion to –217 bp (LEC5) resulted in a four-fold reduction (0.12%). Further reduction of the promoter length (LEC6 and LEC7) resulted in levels of PSL that were below the detection limit. Thus, the sequence between –217 and –210 (CGAAGAA) seems to contain important information for high expression in seed.

PSL was not detectable in leaves or roots of any of the *Psl*-transformed plants.

Progressive 5′ deletions in the Psl *promoter result in a gradual decrease of promoter activity in seeds*

The ELISA method used to detect *Psl* gene expression in tobacco is not very sensitive; PSL levels below 0.01% of total soluble protein cannot be detected. Therefore, another set of constructs was made containing the *Psl* promoter or different 5′ deletion derivatives coupled to the coding region of β-glucuronidase (*gusA*) reporter gene and the terminator of a ribulose-1,5-bisphosphate carboxylase small subunit (*RbcS*-3C) gene from pea (Fig. 2B). These constructs as well as a negative control, containing the *gusA* gene under control of the CaMV 35S TATA box (GusSH-47), were introduced into tobacco by leaf disc transformation, and leaf, root and mature seed of the transgenic plants were analysed for GUS activity. The results are shown in Fig. 4 and summarized in Table 2.

The average GUS activity in leaf tissue of plants containing the 2116 bp *Psl* promoter (LG1) was about 20 pmol MU per minute per mg protein. The shorter promoters that were tested (LG2, LG4 and LG7) resulted in background GUS activities in leaf tissue with levels similar to those with GusSH-47 (2 pmol min^{-1} mg^{-1}). In root, none of the *Psl* constructs had activity above the background value found in untransformed plants. The higher values obtained for GusSH-47 are

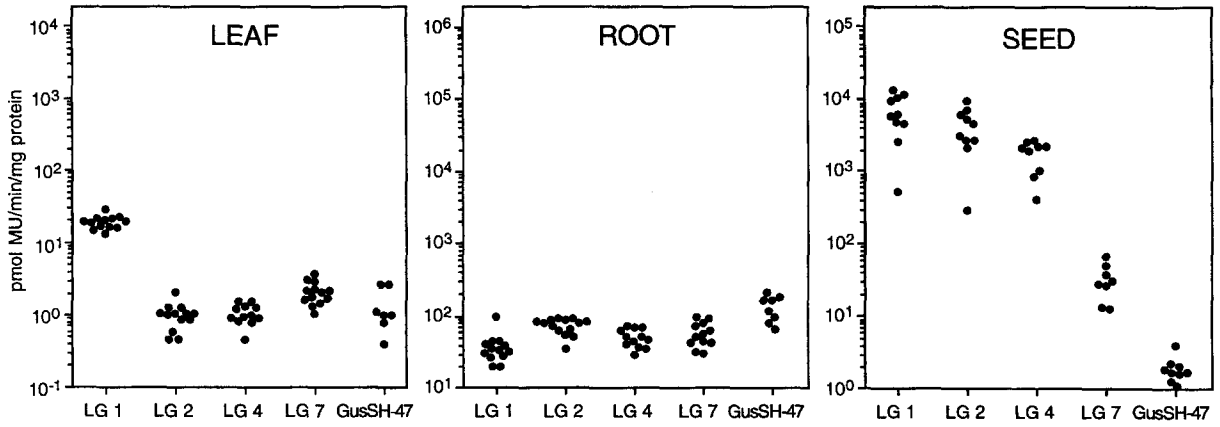

Figure 4. Progressive 5′ deletions in the *Psl* promoter result in a gradual decrease of promoter activity in seeds. GUS activity in transgenic tobacco plants transformed with the chimeric *Psl* promoter-*gusA* genes LG1, LG2, LG4 and LG7. For comparison, plants transformed with a *gusA* gene under control of the TATA box of the CaMV 35S promoter (GusSH-47) were analysed. GUS activity was determined in leaf, root and seed extracts. Note the logarithmic scales.

Table 2. Mean values of GUS activity (pmol MU per minute per mg protein) measured in extracts of transgenic tobacco plants transformed with the *Psl* promoter constructs listed below. The number of independent transformants analysed are indicated in parenthesis.

Construct	Seed	Leaf	Root
Untransformed	1.0	0.1	78
GusSH-47	2.0 (9)	1.2 (8)	136 (8)
LG1 (–2116)	7125 (10)	19.3 (13)	37 (10)
LG2 (–469)	4469(10)	2.2 (15)	73 (14)
LG4 (–236)	1806 (9)	1.1 (12)	49 (12)
LG7 (– 115)	34 (8)	2.0 (13)	63 (13)
LG2-del	55 (7)		
LG4-del	9.0 (11)		
LG7-del	1.8 (12)		
LG2-mu	2854 (8)		
LG4-mu	828 (10)		
LG7-mu	7.5 (12)		
–46GUS	14 (12)	4.9 (10)	
W1-GUS	41 (6)	25 (4)	
3W1-GUS	1492 (4)	14 (4)	
6W1-GUS	2514 (6)	28 (6)	
9W1-GUS	4308(6)	36 (6)	

probably due to the older age of the roots. Age of the roots was found to influence background GUS activity.

In seeds of LG1-transformed plants GUS activity was very high (7125 pmol min^{-1} mg^{-1}). GUS activity in seeds of LG2 plants, containing the 469 bp *Psl* promoter, was somewhat lower but this difference was not significant. In seeds of LG4 plants, containing the 236 bp *Psl* promoter, GUS activity was about four-fold lower. Further reduction of promoter length to 115 bp strongly reduced GUS activity (34 pmol min^{-1} mg^{-1}). However, this activity was still seventeen-fold higher than that in extracts of GusSH-47 and untransformed seeds. These results show that 115 bp of the *Psl* promoter are sufficient for seed-specific expression. Increasing promoter length strongly enhanced expression in seed.

The 22 bp W1 element is essential for the activity of the Psl *promoter*

Previously, we have shown that a trimer of a 22 bp W1 sequence of the *Psl* promoter confers seed-specific gene expression in transgenic tobacco [31]. This sequence is located between positions –56 to –35 with respect to the transcription start site (Fig. 1). LG7 is the shortest promoter fragment that we have analysed. It contains this W1 element and confers low level seed-specific expression. To determine whether the W1 element is required for seed-specific activity of the *Psl* promoter, it was deleted from the 469 bp, 236 bp and 115 bp promoters, resulting in constructs LG2-del, LG4-del and LG7-del, respectively (Table 1, Fig. 2B). Deletions were introduced in 5′ deletion derivatives and not in the 2116 bp promoter, because the upstream part of the 2116 bp promoter fragment may contain sequences that are functionally similar to W1, thereby

causing redundancy of elements. Furthermore, the 469 bp promoter contains all elements necessary for a high level of seed-specific expression, since the activity of the 469 bp promoter was not significantly different from the activity of the 2116 bp promoter in seed tissues. GUS activity in seeds of tobacco plants transformed with the LG2-del construct was low (Fig. 5 and Table 2), yet was more than 25-fold above background. The 469 bp promoter contains a sequence at –300 that is identical to part of the W1 element (AATGAGTCAT) and is a weak binding site for bZIP proteins [30], which might account for the residual activity. The LG4-del construct showed very little residual activity. Deletion of W1 within the 115 bp promoter (LG7-del) abolished the accumulation of GUS completely. Thus, deletion of the W1 element results in a severe reduction of the activity of the lectin promoter constructs tested.

The W1 element contains a so-called odd base C-box that was found to be a binding site for bZIP proteins [30]. We tested whether this sequence is the essential part of W1 within the *Psl* promoter. Both G's in the sequence ATGAGTCATC and both G's in the opposite strand were found to be important for binding of bZIP proteins [30]. Mutation of these four nucleotides resulted in loss of *in vitro* binding of cloned bZIP [30] and nuclear proteins [31]. These mutations also abolished the expression conferred by a trimer of the W1 element [31]. These four base pairs were mutated in the LG2, LG4 and LG7 promoters (Table 1), resulting in constructs LG2-mu, LG4-mu and LG7-mu, respectively (Fig. 2B). The GUS activities of LG2-mu seeds were not significantly lower compared to LG2, presumably because the upstream W1-like element at –300 masks the four base pair mutation in W1. Seeds of tobacco plants containing LG4-mu and LG7-mu constructs did show lower activity compared with the intact LG4 and LG7 promoters (Fig. 5 and Table 2). These results demonstrate that the bZIP protein-binding site within the W1 element contributes to the activity of the pea lectin promoter, but that other nucleotides within the W1 element are also important.

Activity of the 22 bp W1 element in seed increases with copy number

Three copies of the W1 element confer seed-specific expression onto a heterologous TATA box [31]. However, the expression level of this construct is about 5-fold lower than the expression level of the 2116 bp *Psl* promoter in seed. To test whether the expression level depends on the number of W1 elements, –46GUS

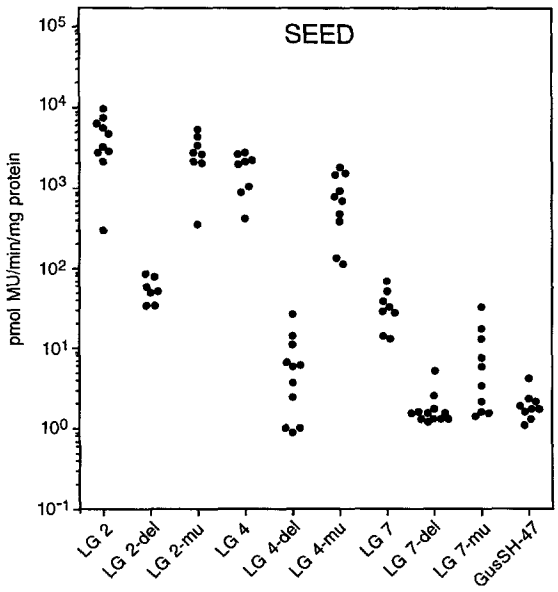

Figure 5. W1 is essential for high activity of the *Psl* promoter in seeds. GUS activity in seed extracts of transgenic tobacco plants transformed with LG2, LG4 or LG7 and derivatives thereof with either a deletion (del) or a 4 bp mutation (mu) in the W1 element. For comparison, plants transformed with GusSH-47 were analysed. Note the logarithmic scale.

derivatives containing 1, 3, 6, or 9 copies of the W1 element were made (Table 1, Fig. 2C). GUS activity was determined in seed and leaf of tobacco transformants (Fig. 6 and Table 2). One copy of W1 slightly increased GUS activity in seed as well as in leaf compared to –46GUS. Three W1 elements resulted in much higher GUS activity in seed, but not in leaf. Six copies of W1 had about the same activity as three copies. GUS activities in seed further increased with 9 copies of the W1 element, but remained low in leaf. These results show that high seed-specific expression can be achieved by multimerization of the W1 element, and that GUS activity is correlated with copy number. Multimerization of the W1 element up to nine copies resulted in high levels of GUS activity like those observed with LG1 and LG2.

Discussion

The *Psl* promoter confers seed-specific expression in tobacco. Comparison of the relative expression levels showed a good correlation between results obtained with *Psl* gene constructs and chimeric *Psl*-promoter-*gusA* constructs. This means that the sequences that

regulate transcription of the *Psl* gene are mainly located in the promoter region. For both sets of constructs no expression in transgenic tobacco roots was detected, whereas low amounts of lectin are present in pea roots [4]. Since in pea the level of expression is more than three orders of magnitude lower in root than in seed [2], it is likely that in transgenic tobacco the expression in roots is below the limit of detection.

The 22 bp W1 element of the *Psl* promoter was previously shown to confer seed-specific expression onto a heterologous TATA box [31]. Here we show that it is necessary for activity of the *Psl* promoter. In addition, nine copies of W1 confer a similar expression level as the intact *Psl* promoter. Taking these results together, it can be concluded that the W1 element plays an important role in the seed-specific activity of the *Psl* promoter.

One copy of the W1 element slightly increases expression in seed as well as in leaf when compared to –46GUS (Fig. 6). Multimerization of the W1 element is necessary to obtain a seed-specific expression pattern. The 236 bp *Psl* promoter confers high seed-specific expression, although it contains only one W1 element. Thus, W1 must act in combination with other enhancing sequences. A good candidate is the sequence located between –217 and –210. Deletion of this sequence greatly reduced promoter activity of the complete *Psl* gene (Fig. 3). The *Psl-gusA* 5′ deletion constructs LG7 (–115) also has severely reduced activity compared to the other constructs (LG1, LG2 and LG4) in which this sequence is still present.

The observation that a promoter element that occurs in a single copy in its natural context only has high activity when multimerized was also made for the *as-1* sequence of the CaMV 35S promoter in leaf. A single *as-1* element has low activity in leaf, but multimerization results in high expression [18]. Like the W1 element, deletion of [5] or point mutations [17] in *as-1* within the 35S promoter decrease the activity in leaf.

Mutation of 4 bp in the bZIP protein binding site located in the W1 element [30] only partly inactivated the *Psl* promoter. However, the same 4 bp mutation completely inactivated the 3W1-GUS construct [31]. A possible explanation for this apparent paradox is that DNA-binding proteins need to be stabilized on the DNA by protein-protein interactions, either between proteins binding to multiple copies of the seed-specific element as in W1 multimers, or between proteins binding to a single copy of the seed-specific element and proteins binding to flanking sequences as in the *Psl* promoter. According to this explanation, in the LG-mu constructs weaker binding of the protein interacting with W1 is overcome by interaction of this protein with other proteins, whereas weak interactive binding to the 3W1 mutant multimer is not sufficiently stable.

The observed expression of the *Psl* promoter in tobacco is similar to the expression of the intact *Psl* gene in pea ([2] and de Pater, unpublished results). Other seed-specific promoters were also found to be similarly active in heterologous plants. This suggests that the molecular mechanisms which lead to seed-specific expression are similar in different plant species. However, similarities in promoter structure found sofar are limited. So-called RY repeats were found to play an important role in several seed-specific promoters. In some promoters the RY repeat has been reported to function as a positive element [1, 7, 11, 20] but in another case as a negative element [6]. A sequence with homology to the RY repeat is also present in the *Psl* promoter (located at –16 to –1), but whether it is functional is unknown. Progressive 5′ deletions in the *Psl* promoter resulted in gradual decrease of the expression in seed. In contrast to some other studies [3, 6, 21, 25, 37, 42], negative elements were not found with the deletions shown in Fig. 1.

The W1 element, which functions as a seed-specific element when multimerized and is indispensible for high activity of the *Psl* promoter, is able to bind the bZIP protein O2 *in vitro* with high affinity [30]. This protein, which is endosperm-specific, is essential for the expression of certain maize endosperm proteins [27]. This suggests that a tobacco O2 homologue is involved in seed-specific expression conferred by W1. The O2-binding sites in promoters of the zein [34], B-32 [22] and *Psl* genes contain ACGT core elements or variations thereof, like the odd-base C-box in W1 [30]. It is not known whether the O2-binding sites present in the zein and B-32 promoters function as seed-specific elements by themselves. Many promoters containing a functional copy of this core element are not seed-specific. Also, a 2 bp mutation in the flanking sequence of a seed-specific ACGT element changes it into a root-specific element [33]. Evidently, sequences flanking the ACGT core determine the tissue-specific activity of this element. Characterization of O2-binding sites and other elements in promoters other than the *Psl* promoter that are both necessary and sufficient for seed-specific expression may answer whether seed-specific promoters have similar seed-specific *cis*-elements such as the ACGT sequence or whether other seed-specific elements exist.

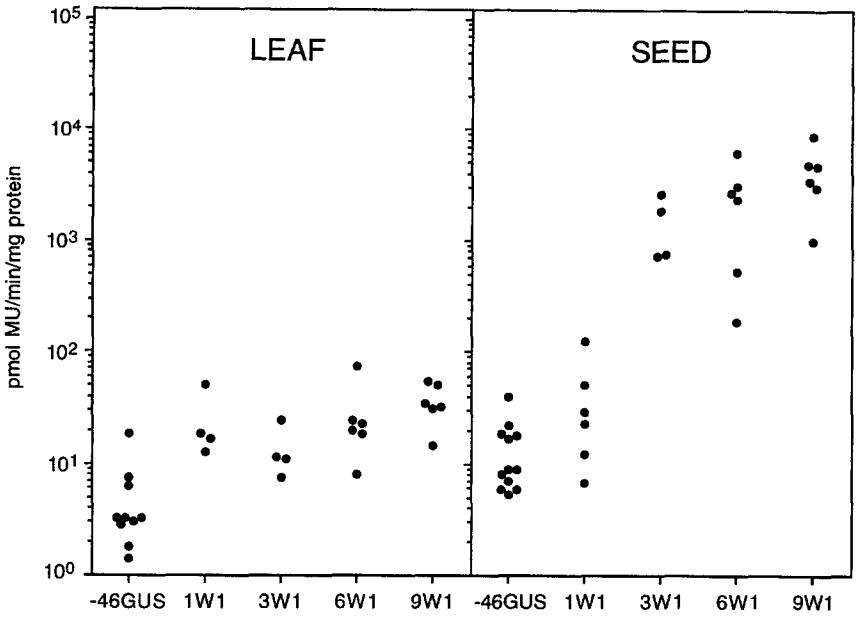

Figure 6. Increasing W1 copy number results in a gradual increase of the expression of a minimal promoter in seeds. GUS activity in leaf and seed extracts of transgenic tobacco plants transformed with a *gusA* gene under control of the TATA box of the CaMV 35S promoter (–46GUS) or derivatives containing 1, 3, 6 or 9 copies of the W1 element. Note the logarithmic scale.

The identification of a promoter element that is necessary and as a multimer is sufficient for expression in seeds has provided a powerful tool for the identification of *trans*-acting factors and other steps in the regulatory pathway leading to seed-specific transcription.

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References

1. Bäumlein H, Nagy I, Villarroel R, Inzé D, Wobus U: *Cis*-analysis of a seed protein gene promoter: the conservative RY repeat CATGCATG within the legumin box is essential for tissue-specific expression of a legumin gene. Plant J 2: 233–239 (1992).
2. Buffard D, Kaminski P-A, Strosberg AD: Lectin-gene expression in pea (*Pisum sativum* L.) roots. Planta 173: 367–372 (1988).
3. Bustos MM, Begum D, Kalkan FA, Battraw MJ, Hall TC: Positive and negative *cis*-acting DNA domains are required for spatial and temporal regulation of gene expression by a seed storage protein promoter. EMBO J 10: 1469–1479 (1991).
4. Díaz CL, Lems-van Kan P, Van der Schaal IAM, Kijne JW: Determination of pea (*Pisum sativum* L.) root lectin using an enzyme-linked immunoassay. Planta 161: 302–307 (1984).
5. Fang R-X, Nagy F, Sivasubramaniam S, Chua N-H: Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1: 141–150 (1989).
6. Fiedler U, Filistein R, Wobus U, Bäumlein H: A complex ensemble of *cis*-regulatory elements controls the expression of a *Vicia faba* non-storage seed protein gene. Plant Mol Biol 22: 669–679 (1993).
7. Fujiwara T, Beachy RN: Tissue-specific and temporal regulation of a βconglycinin gene: roles of the RY repeat and other *cis*-acting elements. Plant Mol Biol 24: 261–272 (1994).
8. Gallusci P, Salamini F, Thompson RD: Differences in cell type-specific expression of the gene *Opaque 2* in maize and transgenic tobacco. Mol Gen Genet 244: 391–400 (1994).
9. Gatehouse JA, Bown D, Evans IM, Gatehouse LN, Jobes D, Preston P, Croy RRD: Sequence of the seed lectin gene of pea (*Pisum sativum* L.). Nucl Acids Res 15: 7642 (1987).
10. Guiltinan MJ, Marcotte WR Jr, Quatrano RS: A plant leucine zipper protein that recognizes an abscisic acid response element. Science 250: 267–271 (1990).
11. Hattori T, Vasil V, Rosenkrans L, Hannah LC, McCarty DR, Vasil IK: The *viviparous-1* gene and abscisic acid activate the *C1* regularory gene for anthocyanin biosynthesis during seed maturation in maize. Genes Devel 6: 609–618 (1992).
12. Hoedemaeker FJ, Richardson M, Díaz CL, de Pater BS, Kijne JW: Pea (*Pisum sativum* L.) seed isolectins 1 and 2 and pea

root lectin result from carboxypeptidase-like processing of a single gene product. Plant Mol Biol 24: 75–81 (1994).
13. Hood EE, Gelvin SB, Melchers LS, Hoekema A: New *Agrobacterium* helper plasmids for gene transfer to plants. Transgen Res 2: 208–218 (1993).
14. Izawa T, Foster R, Nakajima M, Shimamoto K, Chua N-H: The rice bZIP transcriptional activator RITA-1 is highly expressed during seed development. Plant Cell 6: 1277–1287 (1994).
15. Jefferson RA: Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5: 387–405 (1987).
16. Kaminski PA, Buffard D, Strosberg AD: The pea lectin gene family contains only one functional gene. Plant Mol Biol 9: 497–507 (1987).
17. Lam E, Benfey PN, Gilmartin PM, Fang R-X, Chua N-H: Site-specific mutations alter *in vitro* factor binding and change promoter expression pattern in transgenic plants. Proc Natl Acad Sci USA 86: 7890–7894 (1989).
18. Lam E, Chua N-H: GT-1 binding site confers light responsive expression in transgenic tobacco. Science 248, 471–474 (1990).
19. Lam E, Chua N-H: Tetramer of a 21-base pair synthetic element confers seed expression and transcriptional enhancement in responce to water stress and abscisic acid. J Biol Chem 266: 17131–17135 (1991).
20. Lelievre J-M, Oliveira LO, Nielsen NC: 5′-CATGCAT-3′ elements modulate the expression of glycinin genes. Plant Physiol 98: 387–391 (1992).
21. Lessard PA, Allen RD, Fujiwara T, Beachy RN: Upstream regulatory sequences from two β-conglycinin genes. Plant Mol Biol 22: 873–885 (1993).
22. Lohmer S, Maddaloni M, Motto M, Di Fonzo N, Hartings H, Salamini F, Thompson RD: The maize regulatory locus *Opaque-2* encodes a DNA- binding protein which activates the transcription of the *b-32* gene. EMBO J 10: 617–624 (1991).
23. Marcotte WR Jr, Bayley CC, Quatrano RS: Regulation of a wheat promoter by abscisic acid in rice protoplasts. Nature 335: 454–457 (1988).
24. Marcotte WR Jr, Russell SH, Quatrano RS: Abscisic acid-responsive sequences from the Em gene of wheat. Plant Cell 1: 969–976 (1989).
25. Matzke AJM, Stöger EM, Scherthaner JP, Matzke MA: Deletion analysis of a zein gene promoter in transgenic tobacco plants. Plant Mol Biol 14: 323–332 (1990).
26. McCarty DR, Hattori T, Carson CB, Vasil V, Lazar M, Vasil IK: The *viviparous-1* developmental gene of maize encodes a novel transcriptional activator. Cell 66: 895–905 (1991).
27. Motto M, Di Fonzo N, Hartings H, Maddaloni M, Salamini F, Soave C, Thompson RD: Regulatory genes affecting maize storage protein synthesis. Oxf Surv Plant Mol Cell Biol 6: 87–114 (1989).
28. Offringa R, Franke-van Dijk MEI, de Groot MJA, van den Elzen PJM, Hooykaas PJJ: Nonreciprocal homologous recombination between *Agrobacterium* transferred DNA and a plant chromosomal locus. Proc Natl Acad Sci USA 90: 7346–7350 (1993).
29. Pasquali G, Ouwerkerk PBF, Memelink J: Versatile transformation vectors to assay the promoter activity of DNA elements in plants. Gene 149: 373–374 (1994).
30. de Pater S, Katagiri F, Kijne J, Chua N-H: bZIP proteins bind to a palindromic sequence without an ACGT core located in a seed-specific element of the pea lectin promoter. Plant J 6: 133–140 (1994).
31. de Pater S, Pham K, Chua N-H, Memelink J, Kijne J: A 22-bp fragment of the pea lectin promoter containing essential TGAC-like motifs confers seed-specific gene expression. Plant Cell 5: 877–886 (1993).
32. Pla M, Vilardell J, Guiltinan MJ, Marcotte WR, Niogret MF, Quatrano RS, Pagès M: The *cis*-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene *rab28*. Plant Mol Biol 21: 259–266 (1993).
33. Salinas J, Oeda K, Chua N-H: Two G-box-related sequences confer different expression patterns in transgenic tobacco. Plant Cell 4: 1485–1493 (1992).
34. Schmidt RJ, Kedudat M, Aukerman MJ, Hoschek G: Opaque-2 is a transcriptional activator that recognizes a specific target site in 22-kD zein genes. Plant Cell 4: 689–700 (1992).
35. Skriver K, Mundy J: Gene expression in response to abscisic acid and osmotic stress. Plant Cell 2: 503–512 (1990).
36. Skriver K, Olsen FL, Rogers JC, Mundy J: Cis-actng DNA elements responsive to gibberellin and its antagonist abscisic acid. Proc Natl Acad Sci USA 88: 7266–7270 (1991).
37. Stålberg K, Ellerström M, Josefsson L-G, Rask L: Deletion analysis of a 2S seed storage protein promoter of *Brassica napus* in transgenic tobacco. Plant Mol Biol 23: 671–683 (1993).
38. Stubbs ME, Carver JP, Dunn RJ: Production of pea lectin in *Escherichia coli*. J Biol Chem 261: 6141–6144 (1986).
39. Ueda T, Waverczak W, Ward K, Sher N, Kedudat M, Schmidt RJ, Messing J: Mutations of the 22- and 27-kD zein promoters affect transactivation by the Opaque-2 protein. Plant Cell 4: 701–709 (1992).
40. Unger E, Parsons RL, Schmidt RJ, Bowen B, Roth BA: Dominant negative mutants of Opaque 2 suppress transactivation of a 22-kD zein promoter by Opaque 2 in maize endosperm cells. Plant Cell 5: 831–841 (1993).
41. Vancanneyt G, Schmidt R, O'Conner-Sanchez A, Willmitzer L, Rocha-Sosa M: Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet 220: 245–250 (1990).
42. Yamamoto S, Nishihara M, Morikawa H, Yamauchi D, Minamikawa T: Promoter analysis of seed storage protein genes from *Canavalia gladiata* D.C. Plant Mol Biol 27: 729–741 (1995).