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TATA Box and Initiator Functions in the Accurate Transcription of a Plant Minimal Promoter in Vitro

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The functional architecture of the proximal region of a rice phenylalanine ammonia-lyase (*PAL*) promoter was analyzed by transcription of *PAL*-β-glucuronidase (*GUS*) templates by whole-cell extracts of rice cell suspension cultures. The promoter 5′ truncated to position −35 was sufficient for accurate initiation of basal transcription. Substitution of the TATTTAA sequence between positions −35 and −28 with GCGGGTT or 2-bp substitutions to give TCGTTAA and TATGGAA inactivated the minimal promoter. Moreover, the function of the TATTTAA sequence was dependent on its position relative to the initiation site; hence, this element is an authentic TATA box essential for RNA polymerase II–dependent transcription. Substitutions in the TCCAAG initiator *cis* element (−3 to +3) at the −1 (C to A or G) and +1 (A to C or T) residues caused inaccurate initiation, whereas mutations at the other residues of this conserved element or sequence substitutions between the TATA box and initiator had little effect. TATA box and initiator functions were confirmed by analysis of the effects of promoter mutations on expression in stably transformed rice cell suspensions and plants. We concluded that the proximal region of the *PAL* promoter has a simple functional architecture involving a TATA box appropriately positioned upstream of the initiator. Transcription of derivatives of such minimal promoters by highly active cell extracts should allow molecular analysis of functional interactions between specific *cis* elements and cognate *trans* factors.

INTRODUCTION

In eukaryotic genes transcribed by RNA polymerase II, the proximal region of the promoter usually contains an initiator *cis* element that overlaps the transcription start site and a TATA box typically located ~30 nucleotides upstream (Aso et al., 1994; Tjian and Maniatis, 1994). In animal and yeast systems, accurate transcription from many genes is dependent on the TATA box, which directs the formation of a stable initiation complex following binding of transcription factor TFIID (Struhl, 1987; Aso et al., 1994; Buratowski, 1994). Modulation of the formation or stability of the initiation complex by *trans*-acting proteins that bind to distal *cis* elements requires an intact TATA box (Horikoshi et al., 1988a, 1988b; Lin and Green, 1991; Grayson et al., 1995), and in some genes the TATA box region is a determinant of cell- or organ-specific expression (McCormick et al., 1991; Kloeckener-Gruissem et al., 1992). The initiator *cis* element, which has been less studied and has a less well defined consensus sequence than the TATA box (Weis and Reinberg, 1992), binds *trans*-acting factors for the placement of the start site (Means and Farnham, 1990; Roy et al., 1991; Seto et al., 1991; Li et al., 1994; Mukherjee et al., 1995), and in certain TATA-less promoters, this element can itself mediate the initiation of transcription (Smale and Baltimore, 1989; Zenzie-Gregory et al., 1992).

The structural conservation of the plant, human, and yeast TFIID proteins indicates that the basic machinery for RNA polymerase II–mediated transcription is likely conserved in all eukaryotes (Gasch et al., 1990; Vogel et al., 1993). However, although the TATA box sequences of many plant genes closely correspond to the eukaryotic consensus (Joshi, 1987) and the requirement of the TATA box for the transcription of a few plant genes has been confirmed by analysis of promoter mutations in transgenic plants (Morelli et al., 1985; Timko et al., 1985; Chen et al., 1986; Zhu et al., 1993), the functional architecture of the proximal regions of plant promoters has not been studied in detail. Such information is a prerequisite for understanding the molecular interactions underlying the functions of specific transcription factors in the selective regulation of plant promoters by developmental and environmental cues (Goldberg et al., 1989; Katagiri and Chua, 1991).

We have recently described an in vitro transcription system in which the rice phenylalanine ammonia-lyase (*PAL*) *ZB8* gene is accurately transcribed in an RNA polymerase II–dependent reaction by whole-cell extracts of rice cell suspension cultures (Zhu et al., 1995a). This convenient in vitro transcription system provides the basis for a detailed dissection of the functional architecture of plant minimal promoters. We show here that accurate basal transcription of the rice *PAL* gene requires both the TATA box for transcriptional activity and the initiator element for accurate placement of the start site, with the functional interaction between these two *cis* elements being critically dependent on their spacing in the promoter.

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RESULTS

Minimal Promoter for Transcription of the Rice *PAL ZB8* Gene

To determine the minimal 5′ flanking sequence required for transcription of the *ZB8* gene, two sets of constructs containing *ZB8* promoter sequences (Zhu et al., 1995b) with different 5′ deletions fused to the β-glucuronidase (*GUS*) reporter gene were tested for their transcriptional activity. The p81, p54, p35, and p20 constructs depicted in Figure 1A were used for rice transformation by biolistic bombardment of immature embryos to generate transgenic suspension-cultured cells. The basal transcription of the rice *ZB8 PAL* gene in unelicited cells is low, and therefore, poly(A)$^+$ RNA was isolated for primer extension analysis. The levels of *ZB8–GUS* expression in transgenic cells containing the p81 and p54 gene fusions were higher than those in p35 transgenic cells. In p20 transgenic cells, no *GUS* transcripts were detected, indicating that the *ZB8* promoter 5′ truncated at position −20 was inactive in vivo (Figure 1B). Thus, 5′ flanking sequences to position −35 were sufficient for basal transcription of the *ZB8* gene in vivo, and the region between positions −35 and −21, including the putative TATA box located between positions −34 to −28, was required for this basal activity.

We next examined initiation of transcription by these 5′ truncated *ZB8* promoters in vitro by using the p81m, p54m, p35m, and p20m supercoiled plasmids depicted in Figure 2A as templates for transcription by rice whole-cell extracts prepared from suspension-cultured cells, followed by primer extension analysis as previously described (Zhu et al., 1995a). This set of constructs was identical with that used for transformation (see above), except for an internal deletion from position +44 of the *ZB8 PAL* gene to −10 of the *GUS* reporter gene. This deletion was introduced to optimize the primer extension reaction, which is most efficient with products between 60 and 100 nucleotides. p81m and related templates would be expected to give a product of 75 nucleotides for transcripts initiated at the in vivo start site (Zhu et al., 1995a). Consistent with *ZB8* promoter activity in vivo, the promoter 5′ truncations to positions −81, −54, and −35 were accurately transcribed by the RNA polymerase II–dependent in vitro system, but the promoter truncated to position −20 was inactive. Although the promoters extending to −81 and −54 were more active than the 5′ truncation to position −35 (Figure 2B), p35m nonetheless gave accurate initiation of transcription in vitro. These data indicate that 5′ sequences to position −35 are the minimal requirement for transcription initiation both in vivo and in vitro and suggest that the region between −35 and −21, which contains the putative TATA sequence, is important for transcription initiation from this minimal promoter. Given the close correspondence between basal transcription initiation in vivo and in vitro, we decided to employ the convenient, highly active in vitro transcription system to study the functional properties of the TATA box and initiator sequence.

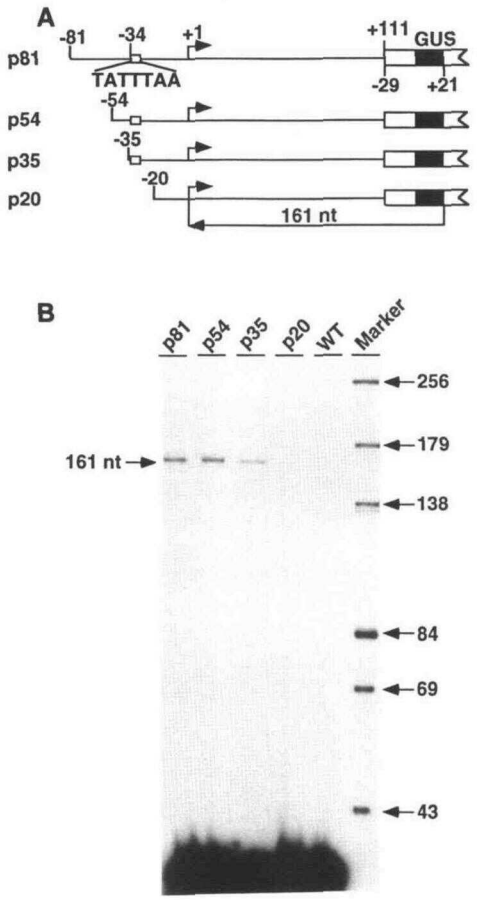


Figure 1. Delineation of the *ZB8* Minimal Promoter in Vivo.

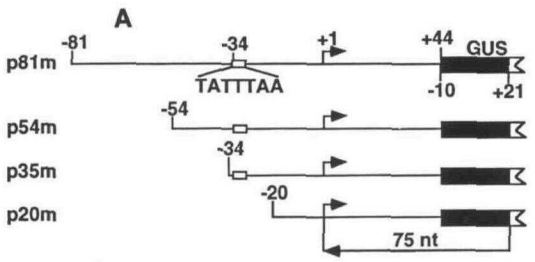
(A) Structures of p81, p54, p35, and p20. The +111 site of the *ZB8* gene with various 5′ flanking sequences was fused 29 bp upstream of the ATG translation initiation codon (the A position of ATG is +1) of the *GUS* gene. The transcription start site is 161 bp from the end of the *GUS-1* primer.

(B) Detection of *GUS* transcripts in transformed suspension-cultured cells. Poly(A)$^+$ RNA isolated from p81, p54, p35, or p20 transgenic suspension-cultured cells and wild-type (WT) cells was used for primer extension analysis with oligonucleotide *GUS-1* as primer. The products of the primer extension reaction were electrophoresed on an 8% polyacrylamide gel containing 7 M urea. The marker lane contains molecular size markers given at right in nucleotides (nt).

TATA Box Requirement for Accurate Initiation of Transcription in Vitro

In higher plants, the TATA box is usually located ~30 bp upstream of the transcription start site (Joshi, 1987), and in the *ZB8* gene there is a TATA box–like motif (TATTTAA) located between positions −34 and −28. This motif closely corresponds to the eukaryotic consensus sequence TATA(T/A)A(T/A) (Breathnach and Chambon, 1981). To confirm the importance of this putative TATA box in the *ZB8* promoter, we made

substitutions by site-directed mutagenesis of this sequence element in the context of the promoter 5′ truncated to position −81. p81m-1, which contains a GCGGGTT 7-bp substitution of the TATTTAA putative TATA box sequence of the parental p81m construct, was essentially inactive as a template for in vitro transcription with rice whole-cell extracts. Moreover, two 2-bp substitutions in which the TATA box was converted to TCGTTAA and TATGGAA sequences in p81m-22 and p81m-23, respectively, likewise resulted in no detectable 75-nucleotide primer extension product in gels loaded with the total reaction products from equivalent in vitro transcription reactions containing the same respective amounts of template and primer (Figures 3A and 3B). Therefore, the TATTTAA sequence in the *ZB8* gene functions as an authentic TATA box essential for



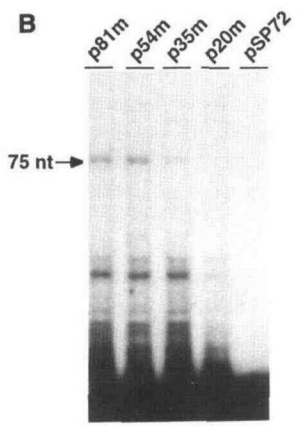


Figure 2. Delineation of the *ZB8* Minimal Promoter in Vitro.

(A) Structures of p81m, p54m, p35m, and p20m. The +44 site of the *ZB8* gene with various 5′ flanking sequences was fused to the −10 site of the *GUS* gene. The transcription start site is 75 bp from the end of the *GUS-1* primer.

(B) In vitro transcription of p81m, p54m, p35m, and p20m templates by rice whole-cell extracts. The transcription reactions were performed in 50 μL containing 0.15 pmol of template DNA, 180 μg of protein, 10 mM potassium acetate (the potassium concentration does not include the 40 mM potassium resulting from the pH adjustment of Hepes buffer with KOH), and 5 mM each of magnesium acetate and magnesium sulfate. The pSP72 vector was used as a control template. nt, nucleotide.

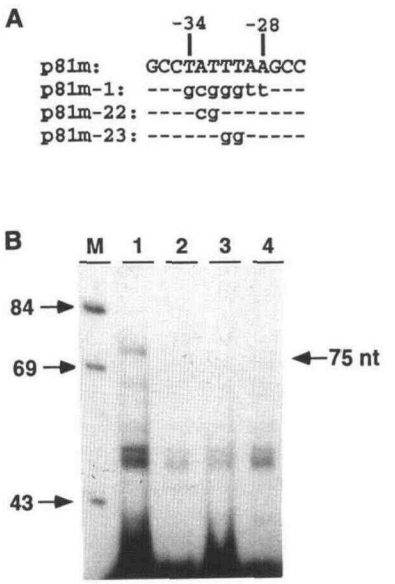


Figure 3. TATA Box Substitutions Abolish the Transcription Initiation Activity of the *ZB8* Promoter.

(A) Sequences around the TATA box in p81m, p81m-1, p81m-22, and p81m-23. Lowercase letters indicate altered residues, and dashes indicate unchanged residues.

(B) In vitro transcription by rice whole-cell extracts of p81m (lane 1), p81m-1 (lane 2), p81m-22 (lane 3), and p81m-23 (lane 4) templates. Lane M contains molecular size markers given at left in nucleotides (nt).

initiation of transcription from the *ZB8* promoter. In contrast, the ~50-nucleotide doublet of primer extension products, which reflect RNA polymerase II–independent transcription (Zhu et al., 1995a), was still observed with templates containing TATA box mutations (Figure 3B).

Initiator Is Required for Accurate Placement of the Transcription Start Site in Vitro

The initiator element, which overlaps the transcription start site, directs the siting of transcription initiation (Weis and Reinberg, 1992; Aso et al., 1994). The sequences around the transcription start site of the *ZB8* gene are highly conserved in all rice *PAL* genes studied so far, and the CA nucleotides located at positions −1 and +1 resemble a typical initiation site for eukaryotic class II genes. To delineate additional aspects of the functional architecture of the *ZB8* promoter, we made a series of substitution mutations in the initiator sequence in the setting of the promoter truncated to −81 (Figure 4A). In vitro transcription initiation assays demonstrated that when the TCCAAG initiator sequence (−3 to +3) was converted to GAACCT, transcripts were generated by the rice whole-cell extracts. However, transcription initiated several base pairs upstream or downstream of the correct start site, resulting in

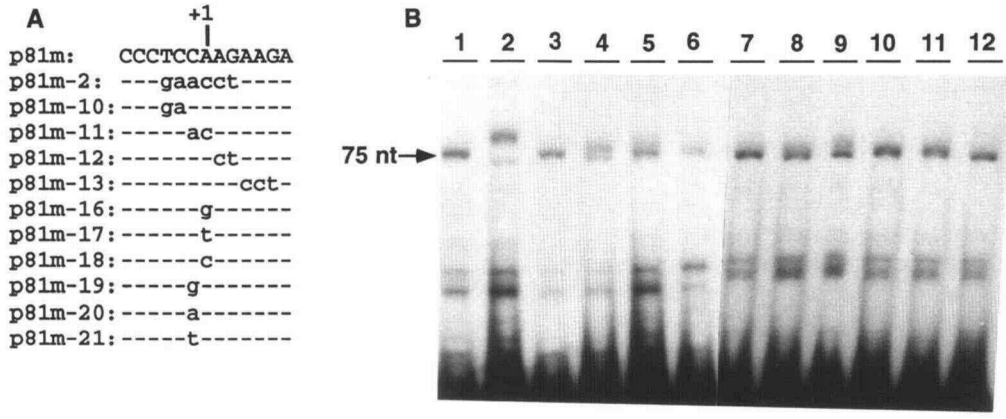


Figure 4. Initiator Sequence Substitutions Alter the Transcription Start Site.

(A) Sequences around the transcription start site in p81m and mutant derivatives. Lowercase letters indicate altered residues, and dashes indicate unchanged residues.

(B) Products from in vitro transcription with rice whole-cell extracts. The templates are as follows: p81m (lane 1), p81m-2 (lane 2), p81m-10 (lane 3), p81m-11 (lane 4), p81m-12 (lane 5), p81m-13 (lane 6), p81m-16 (lane 7), p81m-17 (lane 8), p81m-18 (lane 9), p81m-19 (lane 10), p81m-20 (lane 11), and p81m-21 (lane 12). nt, nucleotide.

two major products, the larger being predominant (Figure 4B). More detailed studies showed that substitutions at positions −2 and −3 (TC to GA), +2 and +3 (AG to CT), or +4 to +6 (AAG to CCT) had almost no effect on the accurate selection of the transcription initiation site. However, if the −1 and +1 nucleotides CA were substituted with AC, transcription of the *ZB8–GUS* gene fusion by rice whole-cell extracts initiated at different sites compared with that observed with the wild-type construct as template (Figure 4B). These results indicate that the −1 and +1 sites of the initiator sequence are particularly important for accurate initiation.

Additional functional analysis using single base-pair substitution mutations showed that pyrimidine-to-purine conversion at the −1 site affected the selection of the transcription initiation site, whereas a C-to-T conversion still resulted in accurate transcription initiation. A-to-G conversion at the +1 site did not affect the selection of the transcription initiation site, whereas conversion from A to a pyrimidine residue (C or T) resulted in inaccurate initiation (Figure 4B). Size differences resulting from initiator element mutations were confirmed by running longer gels (data not shown). These results indicate that the TATA box and the initiator are necessary for transcriptional activity and the correct placement of the initiation site, respectively, and that these two elements function in concert in the context of the promoter 5′ truncated to −81 for accurate, efficient initiation of transcription.

Spacing but Not Nucleotide Sequence between TATA Box and Initiator Is Important for Accurate Transcription Initiation

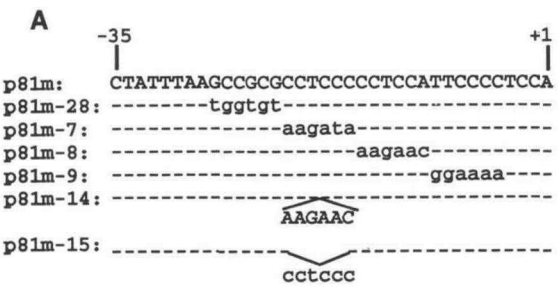
Because the location of the TATA box relative to the transcription start site is relatively conserved in plant genes, it has been suggested that the spacing of the TATA box and initiator is important for determining the initiation site (Joshi, 1987). To test this hypothesis, we made a series of substitution, insertion, and deletion mutations in the region between the TATA box and the initiator sequence (Figure 5A). Deletion (of CCCTCC between positions −21 and −16) or insertion (of AAGAAC between positions −19 and −18) of 6 bp between the TATA box and the initiator eliminated *ZB8* promoter activity in vitro, indicating that the TATA box and initiator could not functionally interact when the spacing between these two *cis* elements was altered. Unlike most other plant genes, the sequence between the TATA box and the initiator of *ZB8* is >75% GC. To study the contribution of this GC-rich sequence to transcription initiation, the effects of a series of 6-bp scanning substitutions were analyzed (Figure 5A). These sequence substitutions did not disrupt accurate transcription initiation in vitro (Figure 5B). Thus, the specific nucleotide sequence between the TATA box and initiator may influence the rate of transcription; however, only the relative spacing of the two elements independent of the intervening nucleotide sequence is important for accurate initiation of transcription.

TATA Box and Initiator Are Necessary for Accurate Basal Transcription from the −35 Minimal Promoter in Vitro and in Vivo

We demonstrated that the TATA box and initiator are necessary for accurate transcription initiation in the context of the *ZB8* promoter 5′ truncated to position −81 (Figures 3 and 4). To confirm the functions of the TATA box and initiator in basal level transcription from the minimal promoter, we next examined the effects of the same mutations in the context of the promoter 5′ truncated to position −35, using p35m as the

parental construct. The results using p35m derivative constructs were similar to those obtained with p81m derivatives as templates. Thus, the TATA box substitution GCGGGTT was essentially inactive, as were the 2-bp substitutions TCGTTAA and TATGGAA in the TATTTAA sequence between positions −34 and −28. Substitution of the initiator TCCAAG sequence between positions −3 and +3 with GAACCT resulted in the generation of two transcripts that initiated several base pairs upstream or downstream of the accurate start site (Figure 6). Substitutions in the initiator at the −1 and +1 sites (CA to AC) disrupted accurate initiation, whereas TC to GA at positions −2 to −3, AG to CT at +2 to +3, or AAG to CCT at +4 to +6 had almost no effect on the placement of transcription initiation in vitro (Figure 6). An insertion or deletion of 6 bp between the TATA box and initiator eliminated accurate basal transcription of the *ZB8* minimal promoter, but substitutions in the nucleotide sequence between the TATA box and initiator that maintained the correct spacing had no effect on the accurate initiation of basal transcription (data not shown).

Finally, to confirm the functions of the TATA box and the initiator in transcription initiation in vivo, we transformed rice with p35m-2 and p35m-3, which contain initiator and TATA box substitutions, respectively. For each construct, total RNA isolated from five independent transgenic plants was analyzed by primer extension using the *GUS-1* primer. Figure 7 shows that the TATA box substitution (p35m-3) resulted in no transcripts in vivo and that the initiator element substitution (p35m-2) resulted in transcripts initiating from incorrect sites. Similar results were obtained in parallel primer extension analysis of total RNA from p35m-2 and p35m-3 transgenic cell suspension cultures (data not shown), and these results indicate that key features of the functional architecture of the *ZB8* promoter deduced from the in vitro transcription assays contribute to basal transcription initiation by this promoter in vivo.



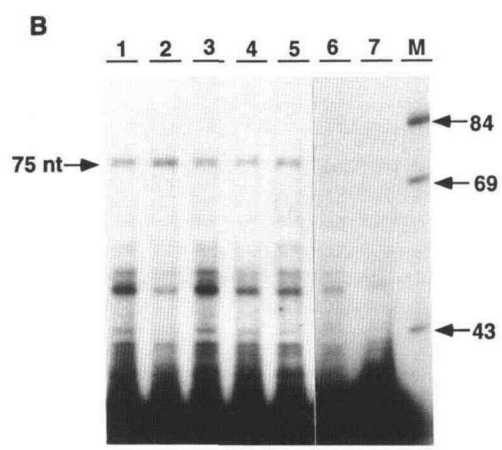
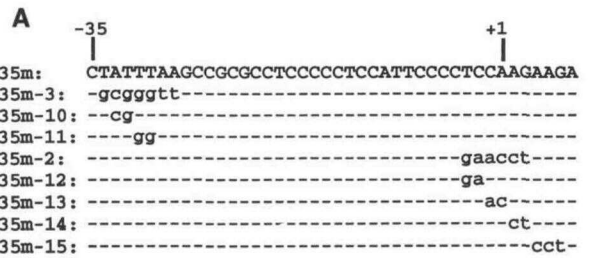


Figure 5. Spacing of the TATA Box and Initiator Is Important for Accurate Initiation of Transcription in Vitro.

(A) Sequences between the TATA box and transcription start site of *ZB8* in p81m and its derivative constructs. Lowercase letters indicate altered residues, and dashes indicate unchanged residues.

(B) In vitro transcription reaction products with p81m (lane 1), p81m-28 (lane 2), p81m-7 (lane 3), p81m-8 (lane 4), p81m-9 (lane 5), p81m-14 (lane 6), and p81m-15 (lane 7) as templates. Lane M contains molecular size markers given at right in nucleotides (nt).



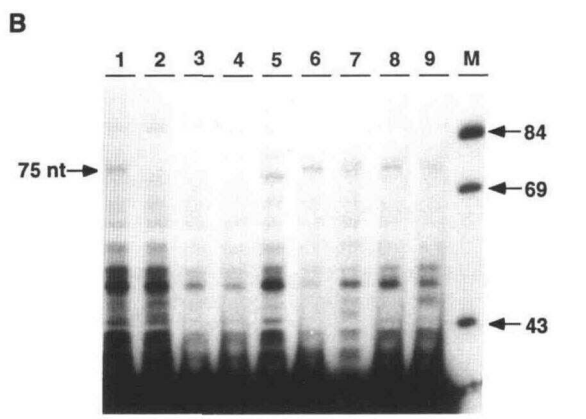


Figure 6. Roles of the TATA Box and Initiator Element in Basal Transcription Initiation.

(A) TATA box and initiator sequences in p35m and mutant derivatives. Lowercase letters indicate altered residues, and dashes indicate unchanged residues.

(B) In vitro transcription reaction products. Templates are p35m (lane 1), p35m-3 (lane 2), p35m-10 (lane 3), p35m-11 (lane 4), p35m-2 (lane 5), p35m-12 (lane 6), p35m-13 (lane 7), p35m-14 (lane 8), and p35m-15 (lane 9). Lane M contains molecular size markers given at right in nucleotides (nt).

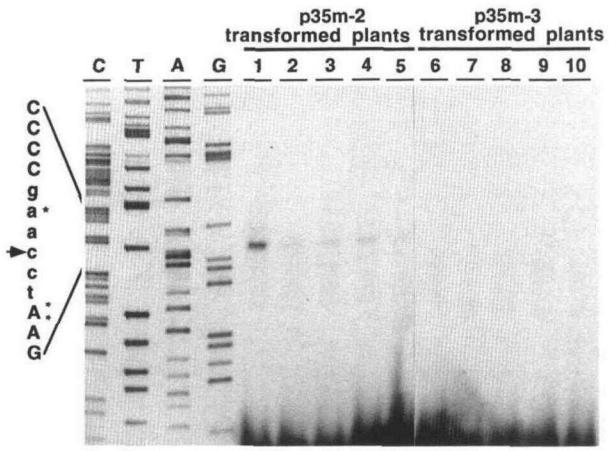


Figure 7. Detection of *GUS* Transcripts in Rice Plants Transformed with p35m-2 and p35m-3.

GUS transcripts in total RNA isolated from transgenic rice plants were analyzed by primer extension using the *GUS-1* primer, and products were compared with those in nucleotide sequence reactions, with p35m-2 as the template (lanes G, A, T, and C). Lanes 1 to 5 contain RNA isolated from p35m-2–transformed plant lines 1 to 5, respectively. Lanes 6 to 10 contain RNA isolated from p35m-3–transformed plant lines 1 to 5, respectively. A double asterisk indicates a strong initiation site; a single asterisk indicates a weak initiation site. The arrowhead indicates the correct transcription start site. Initiator substitution sequences are shown in lowercase letters.

DISCUSSION

We have examined the functional architecture of the proximal region of a rice *PAL* promoter by analysis of the transcription of *PAL* promoter–*GUS* templates by whole-cell extracts of rice cell suspension cultures. This homologous in vitro transcription initiation system gives accurate RNA polymerase II–dependent transcription from the start site used in vivo (Zhu et al., 1995a). Moreover, key findings from these in vitro experiments were confirmed by analysis of the effects of well-characterized promoter mutations on the expression of *PAL–GUS* gene fusions in transgenic rice cell suspensions and plants. Our data demonstrate that the minimal *PAL* promoter requires the TATA box for transcriptional activity and the initiator element for correct placement of the transcription start site, with the spacing but not the nucleotide sequence between these two *cis* elements being critical for their functional interaction.

The TATTTAA sequence between positions −34 and −28 of the rice *PAL ZB8* gene resembles the consensus sequence for putative TATA boxes in plant promoters (Joshi, 1987), and genetic analysis has demonstrated that the specific TATTTAA sequence can function as a TATA box in yeast (Singer et al., 1990). Binding of TFIID to the TATA box initiates assembly of the preinitiation complex, and an Arabidopsis TFIID can substitute for the human protein to mediate transcription from the adenovirus major late promoter by HeLa cell extracts (Mukumoto et al., 1993). TFIID recognizes the TATA box primarily through contact with the minor groove of duplex DNA, and the substitution of the TATA box with GCGGGTT alters the structure of the minor groove to prevent binding of the *trans* factor. In this study, substitution of the rice *PAL* TATA box with this sequence completely blocked transcription from the promoter both in vitro and in vivo. Similarly, the dinucleotide substitutions of AT at positions −33 and −32 and TT at positions −31 and −30 with CG and GG, respectively, which also inactivated the *PAL* promoter, might likewise be expected to alter the surface conformation of the minor groove and, hence, disrupt TFIID binding. Moreover, the function of the TATTTAA sequence in the *PAL* promoter was dependent on its position relative to the initiator because insertion or deletion of 6-bp sequences abolished TATA box–dependent transcription. Hence, the TATTTAA sequence between positions −34 and −28 in the rice *PAL* promoter functions as an authentic TATA box essential for RNA polymerase II–dependent transcription.

Like most plant genes, transcription of the *ZB8* gene initiates at an adenine residue (Zhu et al., 1995b), and the TCCAAG sequence is found between positions −3 and +3 in all rice *PAL* genes examined (Minami et al., 1989; Minami and Tanaka, 1993; Zhu et al., 1995b). This initiator element did not support transcription in the absence of the TATA box, and substitution with GAACCT did not abolish transcription but rather caused initiation at adjacent incorrect sites. Hence, the rice *PAL* initiator element belongs to the class of "weak" eukaryotic initiators, which determine the correct placement of the initiation site but cannot themselves direct the assembly of the transcription complex (O'Shea-Greenfield and Smale, 1992; Weis and Reinberg, 1992; Aso et al., 1994), unlike the "strong" initiators found in TATA-less promoters (Smale and Baltimore, 1989; Zenzie-Gregory et al., 1992; Martinez et al., 1994). Within the *PAL* initiator, a pyrimidine at position −1 and a purine at +1 appear to be critical for accurate initiation of transcription. These residues presumably interact with a *trans*-acting factor in the preinitiation complex, because, as in other eukaryotic systems, the spacing between the TATA box and the initiator is critical, with transcription being abolished by insertion or deletion of 6-bp sequences that alter by one-half turn of the helix the relative orientation of these two *cis* elements. For example, TFIIB may facilitate translocation of DNA duplex melting nucleated by TFIID at the TATA box, and TFIIH and TFIIE are thought to be involved in the formation of an open DNA structure for placement of the initiation site for RNA polymerase II (Schaeffer et al., 1993; Goodrich and Tjian, 1994; Holstege et al., 1995).

From the analysis of 6-bp linker scanning mutations through the −34 to +3 region of the *PAL* promoter, only substitution of the TATA box abolished transcription and only substitution of the initiator element disrupted accurate initiation. The other sequence substitutions, although in some cases causing relatively minor quantitative effects, neither abolished transcription nor caused inaccurate initiation. Hence, the proximal region of the *PAL* promoter has a simple functional architecture

involving a TATA box appropriately positioned upstream of the initiator element. It would be interesting to determine whether this represents a prototype for the minimal promoters of inducible plant genes.

In vitro transcription from the tobacco β-1,3-glucanase and the cauliflower mosaic virus 35S promoters by nuclear extracts of protoplasts derived from tobacco BY-2 suspension-cultured cells has recently been described (Fan and Sugiura, 1995). In contrast, transcription of the ribulose bisphosphate carboxylase small subunit *rbcS-3C* gene, whose expression is tissue specific and light inducible, requires the addition of a nuclear extract from light-grown seedlings. Although this system may prove useful for defining factors specifically required for *rbcS-3C* expression, transcription from this promoter by the augmented extract required templates containing extensive 5′ sequences. Only a weak signal was observed with the promoter 5′ truncated to −351, and no authentic transcript was generated with the promoter truncated to −174. In contrast, the rice whole-cell extract, which in standard reactions supports approximately four cycles of transcription (Zhu et al., 1995a) compared with ~1.5 cycles with the tobacco BY-2 nuclear extract (Fan and Sugiura, 1995), was sufficiently active to drive transcription of the minimal −35 *PAL* promoter, albeit at a somewhat lower rate than with the promoter containing upstream sequences to position −81. Moreover, in this system, transcription of a rice tungro bacilliform virus promoter is stimulated by the addition of a novel recombinant rice transcription factor to whole-cell extracts that have been depleted of endogenous *trans*-acting factors binding to the cognate *cis* element (Y.-H. Yin, Q. Zhu, C. Lamb, and R.N. Beachy, unpublished data), similar to TGA1a stimulation of transcription by a wheat germ extract (Yamazaki et al., 1990). Hence, transcription of derivatives of the rice *PAL* minimal promoter and other defined plant promoters by highly active plant cell extracts, such as the rice suspension culture whole-cell extract, may be suitable for in vitro resection of the combinatorial *cis* element/*trans* factor interactions underlying selective gene expression in response to developmental or environmental signals (Katagiri and Chua, 1991; Brunelle and Chua, 1993).

METHODS

Plant Material

Rice (*Oryza sativa* cv IR54) suspension-cultured cells were a generous gift of Thomas Hodges (Purdue University, West Lafayette, IN) and were maintained as previously described (Zhu et al., 1995a). Rice (cv Taipei 309) cell suspension cultures were developed according to the method of Peng et al. (1990).

DNA Templates

p81, p54, p35, and p20 are pSP72 plasmids containing rice phenylalanine ammonia-lyase (*PAL*) *ZB8* 5′ sequences from positions −81, −54, −35, and −20, respectively, to +111 fused 29 bp upstream of the ATG translation initiation codon of the β-glucuronidase (*GUS*) reporter gene (Jefferson, 1987). p81m, p54m, p35m, and p20m are identical with p81, p54, p35, and p20, respectively, except that they contain an internal deletion from position +44 of the *ZB8 PAL* gene to position −10 of the *GUS* reporter gene. p81m and p35m constructs were used as parental constructs for in vitro mutagenesis (Sambrook et al., 1989). Briefly, single-stranded DNA templates were annealed with oligonucleotides containing appropriate mutations, second strand DNA was synthesized using T4 DNA polymerase, and the gaps were repaired with T4 DNA ligase. The constructs containing mutations were confirmed by nucleotide sequence analysis. The *GUS-1* oligonucleotide (5′-GGTTTCTACAGGACGTAACATAAGGGACTGA-3′) is a synthetic 31-mer identical with the *GUS* antisense DNA strand of pBI101 (Jefferson, 1987) from positions −10 to +21 relative to the translation initiation codon. Assuming transcription is initiated at the correct site, the length of primer extension products using *GUS-1* as a primer would be 161 nucleotides with p81, p54, p35, and p20 constructs and 75 nucleotides with p81m, p54m, p35m, and p20m constructs.

Nucleic Acid Analysis

Genomic DNA from suspension-cultured rice cells and mature leaf tissue was extracted as previously described (Zhu et al., 1995b). Plasmid DNA was isolated by standard methods (Sambrook et al., 1989). RNA from cell suspension cultures and plant tissues was prepared by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1989). Poly(A)$^+$ RNA was isolated by oligo(dT) chromatography (Aviv and Leder, 1972). Nucleotide sequences were determined by the dideoxy chain termination method (Sambrook et al., 1989). In vitro transcription reactions and primer extension analysis of the transcription products were performed as previously described (Zhu et al., 1995a). In each experiment, the respective amounts of template and primer were the same in each reaction, and the total reaction products were loaded in the corresponding lane for gel electrophoresis.

Rice Transformation

Immature rice (cv Taipei 309) embryos were used for biolistic transformation (Li et al., 1993; Zhu et al., 1995b). Briefly, immature embryos were bombarded with tungsten particles coated with p35m-2 or p35m-3 together with pMON410 (Rogers et al., 1986), which contains a cauliflower mosaic virus 35S promoter–hygromycin phosphotransferase gene fusion. The bombarded embryos were plated on N6 media (Chu et al., 1975) containing hygromycin B (30 μg/mL) for 2 weeks. The putative transformed calli were then further selected with hygromycin B at 50 μg/mL for an additional 2 to 3 weeks. Transgenic suspension-cultured cells were developed from these hygromycin-resistant calli according to the method of Peng et al. (1990). For regeneration of transgenic rice plants, hygromycin-resistant calli were cultured on abscisic acid media (5 μg/mL abscisic acid, 2 μg/mL benzaminopurine, and 1 μg/mL naphthaleneacetic acid) for 1 to 2 weeks and then cultured on regeneration media (3 μg/mL benzaminopurine and 0.5 μg/mL naphthaleneacetic acid). The regenerated plants were grown on Murashige and Skoog media (Murashige and Skoog, 1962) containing 50 μg/mL hygromycin B. The transgenic plants were confirmed by polymerase chain reaction analysis.

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