Note

Transcriptional Activities of a Winged Bean Kunitz Chymotrypsin Inhibitor Gene Promoter in Stable and Transient Expression Systems

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Sequential deletions of the promoter region of the *WCI-3b* gene, which encodes the major chymotrypsin inhibitor of winged bean, were constructed and their expression was analyzed in transgenic tobacco plants and in bombarded winged bean seeds. In transgenic tobacco plants, a critical promoter region which is important for high levels of expression in seeds was identified, but deletion of this region had essentially no effect when bombarded into winged bean seeds.

Winged bean (*Psophocarpus tetragonolobus* [L.] DC.) Kunitz-type chymotrypsin inhibitor (WCI) is encoded by a multigene family,13 and the *WCI-3b* gene encodes a major WCI protein. WCI accumulates abundantly in seeds and tuberous roots of winged bean plants.11 In seeds, the *WCI-3b* mRNA accumulates transiently at the mid-maturation stage.1,3 For the analysis of the promoter region, a transient assay using electroporation is an effective system if the transcription of the gene is regulated in the same manner as in *plants*. Protoplasts are widely used for electroporation-mediated transient expression.3,7 However, seeds at mid-maturation stage have a high content of starch which makes protoplast preparation extremely difficult.8 Moreover, genes electroporated into protoplasts are sometimes expressed in a non-regulated manner.9,10 Particle bombardment11 is advantageous over electroporation in gene transfer because intact tissues can be used. Transient expression analyses using particle bombardment have been successfully used to identify cis-elements in several plant gene promoters.12–14 In this communication, we used both particle bombardment of winged bean seeds and *Agrobacterium*-mediated transformation of tobacco plants for the analyses of *WCI-3b* gene expression.

We constructed two sets of chimeric genes in which various lengths of the 5′ upstream region of the *WCI-3b* gene were fused to reporter genes, and compared their transcriptional activities in bombarded winged bean seeds and transgenic tobacco seeds. The 5′ upstream region of the *WCI-3b* gene was deleted sequentially from the 5′ end and fused to the firefly luciferase (LUC) (Fig. 1A) or the β-glucuronidase (GUS) gene (Fig. 1B). The LUC and GUS constructs were used for particle bombardment experiments and transgenic tobacco experiments, respectively.

Figure 2A shows the results of particle bombardment experiments with winged bean seeds 35–40 days after flowering (DAF). At this time, WCI mRNA accumulation peaks.1,1 To normalize the efficiency of DNA introduction in particle bombardment, pBl221,16 which carries the cauliflower mosaic virus 35S promoter fused to the GUS gene, was co-bombarded with the *WCI-3b* gene promoter-LUC constructs, and the *WCI-3b* gene promoter activity was expressed as a ratio of LUC activity to GUS activity. The LUC activity decreased to 50% upon deleting the region of −623 bp to −377 bp, suggesting that this region carries important genetic information for the expression in bombarded winged bean seeds. Figure 2B shows the results of transgenic tobacco experiments. Seeds at 25 DAF (near maturity) were harvested and the GUS activity was measured. There was a drastic reduction in GUS

![Fig. 1. Structure of Fusion Genes Used in Particle Bombardment and Transgenic Experiments.](image-url)

(A): Structure of fusion genes used for transient expression analysis by particle bombardment experiments in winged bean seeds. The promoter fragment of the full length construct (−1.9 kb to +34 bp relative to transcription start site) was deleted sequentially from the 5′ end by exonuclease III using a Kilo-Sequence Deletion kit (Takara Shuzo, Kyoto, Japan). These promoter variants were fused to the LUC gene of a promoter-less vector, pNP-L, which was derived from pD0432.15 The TATA sequences are indicated by the filled boxes. Arrows indicate the transcription start sites and the direction of transcription.

(B): Structure of fusion genes used for stable expression analysis in transgenic tobacco seeds. Construction of the promoters were the same as in (A). The sequentially deleted promoters, except for the −91 bp-promoter, were fused to the GUS gene of pBl101.14

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Abbreviations: DAF, days after flowering; GUS, β-glucuronidase; LUC, luciferase; WCI, winged bean chymotrypsin inhibitor(s).
activity when the region of $-822$ bp to $-623$ bp was deleted. There was also a pronounced reduction in GUS activity when the regions of $-623$ bp to $-377$ bp, and $-377$ bp to $-194$ bp were deleted. On the other hand, GUS activities were very low in leaves in all the constructs, suggesting that the seed-specific pattern of expression is conserved (data not shown).

The $-623$ bp to $-377$ bp region seems to be involved in the transcriptional activation in both systems (Fig. 2). This result suggests that this region carries a cis-element(s) which is important in both systems. However the results obtained from the two systems were different in some other points. The most striking difference was observed concerning the region of $-822$ bp to $-623$ bp. In transgenic tobacco plants, deletion of this region caused a drastic reduction of GUS activity (Fig. 2B, compare pWCI-3b882G and pWCI-3b623G). On the other hand, in the transient assay, the difference in the expression level between pWCI-3b882L and pWCI-3b623L was not so drastic (Fig. 2A). These results indicate that in transgenic tobacco, an important cis-element(s) resides in the region between $-822$ bp and $-623$ bp, but this region is not of such importance in the transient expression system.

A difference was also observed concerning the 3' proximal region of the promoter. In transgenic tobacco seeds, the pWCI-3b77 showed higher GUS activity than the promoter-less construct (pBI101), and this activity was eliminated by further deleting to $-194$ bp (Fig. 2B). In transient expression, however, pWCI-3b377L, pWCI-3b194L, and pWCI-3b91L showed no obvious differences in LUC activity (Fig. 2A). LUC activity of all three of these constructs was higher than that of the control construct, which has no promoter (pNP-L). These results indicate that cis-element(s) required for the minimal transcriptional activity in transgenic tobacco seeds is present between $-377$ bp and $-194$ bp, but this element(s) is not determinable using transient expression in bombarded winged bean seeds. These data indicate that expression of WCI-3b gene integrated into the tobacco genome and transferred by particle bombardment into winged bean seeds are regulated by different modes.

There are several possibilities to account for the differences between the two systems. One possibility is that this difference may reflect the different regulatory mechanisms of gene expression between winged bean seeds and tobacco seeds. Although the transcription of the WCI-3b gene promoter was organ-specific in transgenic tobacco, the cis-element(s) might be different from that required in winged bean plants. Another possibility is that the difference can be ascribed to the chromatin-free structure of plasmid DNA used in the transient system. McPherson et al.\(^{20}\) reported that precise nucleosomal positioning is necessary for the correct formation of nuclear factor complex in the tissue-specific gene promoter of mouse. Regulation of chromatin structure may play a role in the activation of seed protein genes. A third possibility is that the difference in gene dosage between the two systems caused the discrepancy. Since an excess of plasmids is thought to be introduced locally in particle bombardment, these may titrate out the trans-acting factor(s), which is present in a limited amount, and lower the number of active transcriptional complexes.

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