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Duplication of CaMV 35*S* Promoter Sequences Creates a Strong Enhancer for Plant Genes

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A variant of the cauliflower mosaic virus 35*S* promoter with transcriptional activity approximately tenfold higher than that of the natural promoter was constructed by tandem duplication of 250 base pairs of upstream sequences. The duplicated region also acted as a strong enhancer of heterologous promoters, increasing the activity of an adjacent and divergently transcribed transferred DNA gene several hundredfold, and to a lesser extent, that of another transferred DNA gene from a remote downstream position. This optimized enhancer element should be very useful for obtaining high levels of expression of foreign genes in transgenic plants.

DICOTYLEDONOUS PLANTS CAN BE genetically transformed by the transfer of DNA from *Agrobacterium tumefaciens* through the mediation of modified Ti plasmids (*1–4*). Genetic transformation can be used to study the molecular details of plant biology and to create plants with improved or novel characteristics. For many of these purposes, high levels of expression of the transferred genes are necessary. Cauliflower mosaic virus (CaMV) has a double-stranded DNA genome within which two distinct promoters, producing 19*S* and 35*S* transcripts, have been identified (*5, 6*). The 35*S* promoter is constitutively active in several different species (*7*) and so has been used to express a number of foreign genes in transgenic plants (*8–11*). Correct initiation of transcription from the 35*S* promoter is dependent on proximal sequences that include a TATA element, whereas the rate of transcription is determined by sequences that are dispersed over 300 bp of upstream DNA (*7*). We sought to raise the efficiency of transcription of the natural CaMV 35*S* promoter by duplicating the transcription-activating sequences upstream of the TATA element (Fig. 1). We also investigated the effect of the CaMV 35*S* upstream sequences on the expression of several genes and showed that both the natural and duplicated regions act as transcriptional enhancers.

The natural and duplicated 35*S* promoters were transferred to tobacco plants for functional analysis by the use of intermediate vectors derived from pMON178. This vector is a modification of pMON129 (*12*) that contains a nopaline synthase (NOS) gene; a chimeric neomycin phosphotransferase type II (NPTII) gene, which permits selective growth of transformed plant tissue in the presence of kanamycin; and an 1800-bp fragment from the transferrable region of an octopine-type Ti plasmid (Fig. 1). The NPTII-encoding sequences of pMON178 are linked to a promoter that was derived from the transferred DNA (T-DNA) NOS gene. This promoter was excised and replaced by 35*S* promoters with single or double copies of the upstream region to make the intermediate vectors pCKR1 or pCKR2 as shown in Fig. 1.

The intermediate vectors were established in *A. tumefaciens* by homologous recombination with resident copies of the disarmed octopine-type Ti plasmid pTiB6S3-SE as described previously (*13*). The recombinant plasmids contain the octopine-type T-DNA genes 5 and 7 (*14*) and the NPTII and NOS genes of the intermediate vector, all of which are flanked by right and left T-DNA border sequences, which delineate the region of the Ti plasmid that will be integrated into the genome of transformed plant cells (Fig. 1).

Leaf disks of *Nicotiana tabacum* cv. Xanthi H38 were infected with cultures of recombinant *A. tumefaciens* and cultured in vitro as described (*13*). Tobacco cells carrying stably integrated copies of the NPTII gene and associated DNA were selected by growth of calli and subsequent shoot development in the presence of kanamycin (300 μg/ml). Individual shootlets were excised from the transformed tissue and grown to a height of 40 to 60 cm prior to nucleic acid extraction from several leaves (four per plant) at approximately the same stage of development.

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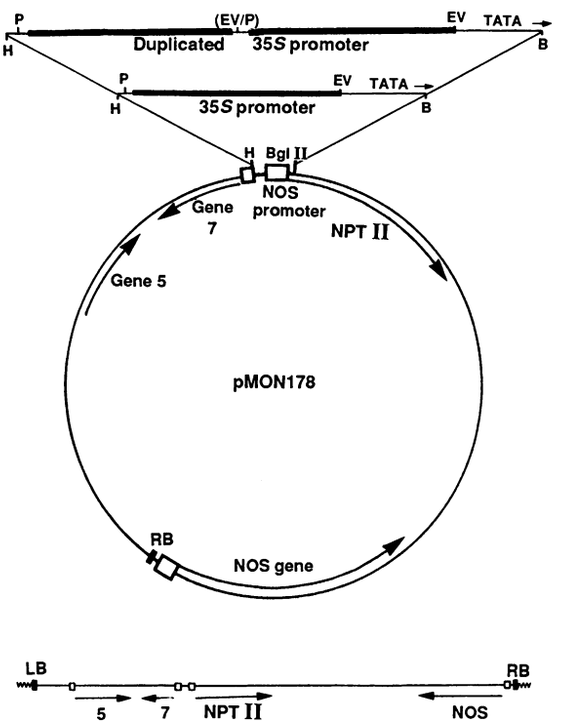


Fig. 1. Structures of genes in intermediate vectors and in transgenic plants. A fragment of the 35*S* promoter extending between positions −343 and +9 was previously constructed in pUC13 by Odell *et al.* (*7*). It was excised as a Cla I–Hind III fragment, made blunt-ended with DNA polymerase I (Klenow fragment), and inserted into the Hinc II site of pUC18 to make pCA1. The upstream region of the 35*S* promoter (nucleotides −343 to −90) was excised from this plasmid as a Hind III–Eco RV fragment and inserted into pCA1 between the Hind III and Pst I (blunt) sites to make pCA2. The unrearranged or duplicated 35*S* promoters were excised from pCA1 or pCA2, respectively, as Hind III–Bam HI fragments and inserted between the Hind III and Bgl II sites of pMON178 to make the intermediate vectors pCKR1 or pCKR2. Intermediate vectors were transferred from *Escherichia coli* to *A. tumefaciens* by triparental mating, and those that had integrated into resident Ti plasmids by recombination were selected as described (*13*). The recombination event results in linkage of the intermediate vector sequences to Ti plasmid sequences including the left T-DNA border and a complete promoter for gene 5. After infection of leaf disks, the DNA between the right (RB) and left (LB) border sequences of the recombinant Ti plasmid is transferred to and chromosomally integrated into the plant cells, with the transferred genes arranged as shown (bottom). The open boxes represent promoters and arrows represent transcribed regions. Abbreviations: B, Bam HI; EV, Eco RV; H, Hind III; P, Pst I; and (EV/P), Eco RV/Pst I fusion.

Copy numbers of transferred DNA ranged from one to five copies, and in most cases, only one or two copies were present including the specific examples shown in this study.

The amounts and initiation sites of transcripts of the transferred genes were determined by their protection of single-stranded DNA probes from digestion by S1 nuclease. When linked to an NOS promoter (MON178), the NPTII-encoding sequences were invariably transcribed at very low levels in the leaves of transgenic plants (Fig. 2). Replacement of the NOS promoter by an unrearranged CaMV 35*S* promoter (CKR1) resulted in a large increase (about tenfold on average) in the number of NPTII transcripts (Fig. 2). This corroborates the observations of others on the relative strengths of these two promoters (*9, 10*) although the differences in the 5′ structures of the 35*S*-NPTII and NOS-NPTII transcripts could affect their stabilities. The modified 35*S* promoter with a duplicated upstream region (CKR2) had by far the highest activity, producing roughly 100 times as many NPTII transcripts as did the NOS promoter (Fig. 2).

The promoter of the chimeric NPTII gene is positioned immediately upstream of the divergently transcribed gene 7 in the

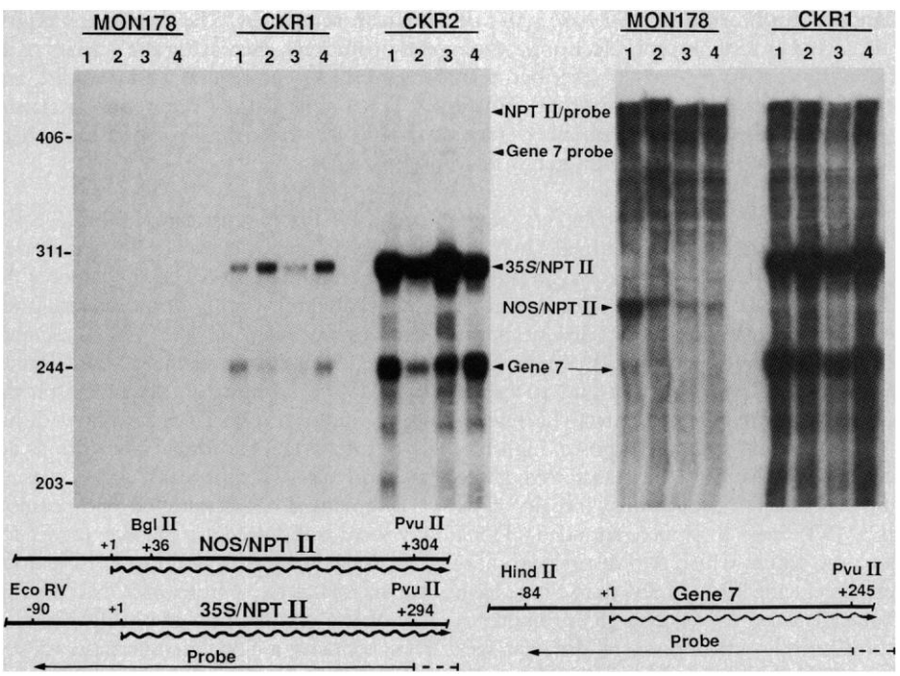


Fig. 2. NPTII and gene 7 transcripts in transgenic plants. Transcripts in total leaf RNA were identified and quantified by S1 nuclease protection. The plants are designated by the name of the intermediate vector used for their transformation and are further identified by individual numbers. The right panel shows a portion of an autoradiograph that was exposed about 20 times as long as that shown in the left panel. The structures of the genes and probes are shown below the autoradiograph. Wavy arrows represent transcripts. Straight arrows represent the probes, with the broken portion derived from M13mp sequences. The positions of full-length probes and probe fragments protected by transcripts are indicated beside the autoradiograph. The NOS/NPTII transcripts are complementary to the 35*S*/NPTII probe only between the Bgl II and Pvu II sites and so protect a shorter probe fragment than the 35*S*/NPTII transcripts. The numbers on the left indicate the positions and lengths in bases of Hpa II fragments of pBR322. The four plants of each transformed type used for this figure are a representative selection of the six to ten plants of each type that were examined for gene expression. Equivalent levels of transcriptional activation by the unrearranged and duplicated 35*S* promoters were observed in additional sets of plants derived from independent transformation experiments. Leaves were ground in a mortar in 25 m*M* EDTA (*p*H 7.5), 75 m*M* NaCl, and 1% SDS, then extracted twice with phenol and once with isobutanol. Liberated nucleic acids were precipitated by the addition of 0.1 volume of 3*M* sodium acetate (*p*H 5.5) and 2 volumes of ethanol. RNA was recovered from the nucleic acid samples by precipitation with 2.5*M* LiCl. Single-stranded, continuously labeled DNA probes were synthesized, hybridized with 2 μg of leaf RNA, digested with S1 nuclease, and analyzed by electrophoresis as described (*35*). The 35*S*/NPTII probe was synthesized from an M13mp18 template containing an insert of the Hind III–Pvu II fragment of the 35*S*/NPTII gene isolated from pCKR1 and was excised from template DNA by digestion with Eco RV. The gene 7 probe was synthesized from an M13mp18 template containing the Hind III–Pvu II fragment of gene 7 isolated from the Hind III Y fragment of the octopine Ti plasmid pTiB6 806 (*20*) and was excised by digestion with Hind III.

transgenic plants (Fig. 1). With an NOS promoter linked to the NPTII-encoding sequences, gene 7 was transcribed at barely detectable levels (Fig. 2). Gene 7 transcription was increased about 40-fold above these levels by the presence of an upstream and divergently oriented 35S promoter, and a further tenfold increase in gene 7 transcription was induced when the upstream region of the NPTII-linked 35S promoter was duplicated. In this situation the upstream region of the 35S promoter appears to serve as a bidirectional promoter-activating element.

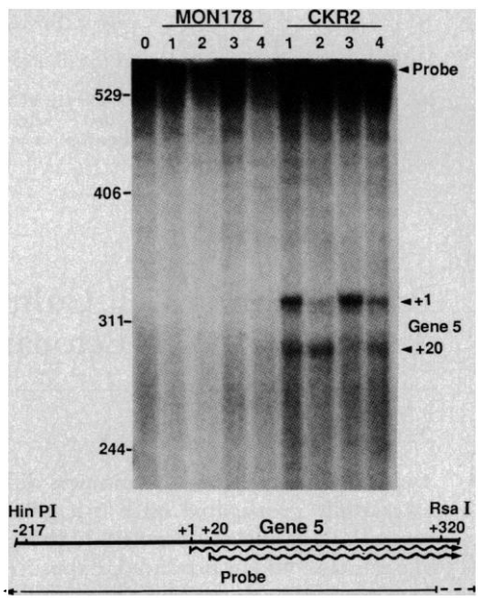
The proportions of NPTII and gene 7 transcripts varied among different plants that had been transformed with the same DNA, even when these two genes were being simultaneously activated by the 35S upstream region. Variability in rates of transcription among either closely linked or widely separated genes within the same fragment of transferred DNA (or even among a pair of transcription start sites associated with a single promoter, gene 5, Fig. 3) is commonly observed in transgenic plants (*8, 15–17*); the causes of such variation in gene expression are unknown.

In addition to activating transcription of the proximal 35S and gene 7 promoter elements, the duplicated upstream region of the 35S promoter was also able to greatly increase transcription at a pair of initiation sites in the gene 5 promoter, despite being located about 2000 bp downstream of it (Fig. 3). Thus, the upstream region of the 35S promoter is analogous to the long-range transcriptional enhancers of animal genes (*18, 19*).

The transcript levels of gene 5 were much lower than those of gene 7 when both were being activated by the duplicated 35S enhancer. This reflects the natural difference in the strengths of their promoters (*14, 20*), but the greater distance between the enhancer and the gene 5 promoter could also result in a lesser degree of transcriptional activation, as observed with other enhancers (*21*).

Transcription of the NOS gene was not significantly affected by the presence of a duplicated 35S enhancer at a remote and downstream location on the same fragment of transferred DNA (Fig. 4). This may result from promoter specificity rather than position or distance effects on the 35S enhancer because insertion of single or duplicated 35S enhancer regions in either orientation between the intact gene 7 and NOS-NPTII gene of pMON178 resulted in strong activation of the gene 7 promoter and only minimal activation of the NOS promoter (*22*).

The activity of the 35S enhancer was



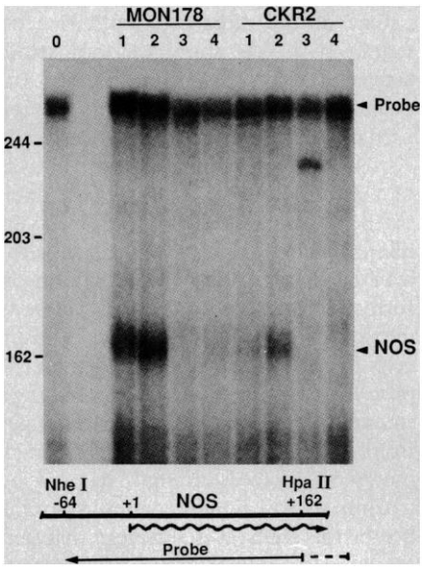


Fig. 3 (left). Gene 5 transcripts in transgenic plants. Transcripts in 2 μg of leaf RNA were identified and quantified by S1 nuclease protection of probes as described in the legend to Fig. 2. Individual plants are designated as in Fig. 2, with "0" indicating an untransformed tobacco plant. Two classes of gene 5 transcripts were identified, initiating about 320 bases (+1) and 300 bases (+20) upstream of the Rsa I site. The probe was synthesized from an M13mp18 template containing an insert of the Hin PI–Rsa I fragment of gene 5 isolated from the Hind III Y fragment of the octopine Ti plasmid pTiB6 806 (*20*) and was excised at the Sst I site in the M13mp18 polylinker beyond the insert. **Fig. 4 (right).** NOS gene transcripts in transgenic plants. Transcripts in 2 μg of leaf RNA were identified and quantified by S1 nuclease protection of probes as described in the legend to Fig. 2. Individual plants are designated as in Fig. 2, with "0" indicating a nontransformed tobacco plant. The probe was synthesized from an M13mp19 template containing an insert of the Eco RI–Hpa II fragment of the NOS gene isolated from pMON178, and was excised by digestion with Nhe I.

increased about tenfold by tandem duplication, indicating that the number of sequence elements within it is a critical determinant of its function. Many animal and viral enhancers contain sets of repeated sequence elements (*19*). Reduction in the number of these repeated elements typically results in a drastic curtailment of enhancer activity (*23, 24*). Exponential increases in transcription over wild type by sequence duplication have not previously been reported. Several short (4- to 7-bp) sequences are repeated within the upstream sequences of the 35S promoter. Among these, the sequence GTGG (and its complement CCAC) is distinguished by being selectively localized within the two promoter regions of the CaMV genome. The 270 bp of DNA upstream from the TATA element of the 35S promoter contains ten copies of these sequences, and the 19S promoter has an additional four copies dispersed over 150 bp of DNA immediately upstream from its TATA element. The remaining 7600 bp in the CaMV genome contain only 26 GTGG sequences (*6*). GTGG sequences are concentrated upstream from or within the promoter regions of many plant genes (*25–30*). A subset of these sequences has attracted attention (*5, 25–28, 31*) because they resemble the sequence GTGTGG$^{AAA}_{TTT}$G, which is common to many of the enhancers associated with viral and animal genes (*32*). Extensive upstream sequences of some light-regulated plant promoters that contain GTGG sequences can activate transcription when positioned in either orientation upstream of a heterologous promoter (*28, 33, 34*). However, such upstream promoter regions excised from the ribulose bisphosphate carboxylase small subunit gene or the light-harvesting chlorophyll a/b–binding protein gene had no significant enhancing effect when placed downstream of a heterologous promoter (*33, 34*). It remains to be established whether the GTGG sequences are responsible for or contribute to the activity of the 35S enhancer and, if so, whether they are functionally analogous to the structurally similar elements of other enhancers and promoters.

We have constructed a modified version of pMON316 (*13*), called pCDX1, which has a duplicated 35S promoter upstream of a polylinker insertion site and a polyadenylation signal sequence. This intermediate vector should be useful for obtaining efficient expression of protein-encoding sequences in transgenic plants. The vector pCKR2 can be used for the same purpose if a complete transcription unit is inserted at the Hind III site of the vector with its promoter adjacent to the 35S enhancer elements.

One of the three promoters that were examined in this study was not strongly activated by the 35*S* enhancer, and other plant promoters may in turn prove to be partially or completely nonresponsive. It is also possible that the pattern of expression of a regulated promoter could be disrupted by the influence of the constitutively active 35*S* enhancer. If loss of regulation is to be avoided, high levels of transcription from such promoters might best be achieved by duplicating the sequence elements that are responsible for their regulated activity. This procedure may be effective for increasing the transcription rates of a wide variety of genes from all life forms, particularly when promoter or enhancer strength has not been maximized by natural processes. As exemplified by this study, it is a strategy that can be successfully applied even when the sequences involved in activating transcription are not fully characterized.

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Propagation in Cell Culture of the Dinoflagellate *Amyloodinium*, an Ectoparasite of Marine Fishes

EDWARD J. NOGA

***Amyloodinium ocellatum*, a common dinoflagellate ectoparasite of marine fishes, was successfully propagated on a fish gill cell line. In vitro infections were similar in cytopathology and development to those reported on natural hosts, and large numbers of parasites could be produced. Exposure of parasites in cell culture to an antiprotozoal drug produced a dose-dependent inhibition of infectivity that was much more sensitive than a motility assay previously used to assess the toxic effects of a drug on protozoan ectoparasites. This propagation system may be a useful model for studying the biology and control of protozoan skin parasites of fishes and for quantitatively studying host-parasite interaction at cellular interfaces.**

ECTOPARASITIC PROTOZOA, MAINLY ciliates and flagellates, commonly infect wild populations of fishes (*1*) and also are important and ubiquitous pathogens in aquaculture (*2*). They damage their hosts by feeding on or within the skin and gill epithelium, which results in osmoregulatory imbalance, respiratory impairment, or secondary infection (*2*). Some ectoparasitic protozoa are facultative commensals capable of a free-living existence, but most are obligate parasites that cannot survive away from the host for an extended period of time (*3–5*). In the latter group is *Amyloodinium ocellatum*, a blastodiniid dinoflagellate that parasitizes warmwater marine and estuarine fishes. Virtually nonspecific in host selection, it is commonly found in natural populations of fishes (*6*) and also is one of the most devastating parasites in warmwater mariculture (*7*).

Amyloodinium has a triphasic life cycle (*8*). In the parasitic stage (Fig. 1) it feeds as a stationary trophozoite (trophont) on the skin and gill surfaces. After several days it detaches, forming a reproductive cyst or tomont on the substrate (Fig. 1). This tomont divides, forming up to 256 free-swimming individuals (dinospores) that can infect a new host (Fig. 1). Under the proper conditions, *Amyloodinium* can complete its life cycle in less than 1 week; it thus can decimate a host population very quickly. Clinical signs of amyloodiniosis include anorexia, hyperventilation, pruritus, and behavioral changes (*6*). Therapy must be directed at the free-swimming infective (dinospore) stage, since dinospores are much less resistant than the tomonts and trophonts (*7, 9*). Infections are asynchronous; thus dinospores do not emerge simultaneously and therapeutic drug levels must be maintained for at least several days to be effective.

Studies of *Amyloodinium* and other ectoparasitic protozoa have been hindered by the lack of in vitro culture systems. This report describes the continuous propagation of *Amyloodinium* in cell culture, in which the entire life cycle of a protozoan ectoparasite of fish has been supported in vitro.

Amyloodinium ocellatum strain DC-1 was originally isolated from aquarium-reared clown fish (*Amphiprion ocellaris*) and has been serially passaged since 1981 on clown fish and hybrid striped bass (*Morone saxatilis* and *M. chrysops*) (*10*). Studies (*11*) had shown that microbial contaminants could be eliminated from harvested parasites by serially transferring individual tomonts through sterile artificial seawater that contained antibiotics. The dinospores that emerged from these tomonts could infect and be serially propagated on gnotobiotic fish. Some parasites in this germ-free system continued to grow and develop even after the death of their host fish (*11*), suggesting that *Amyloodinium* might be adaptable to cell culture.

To test this hypothesis, approximately 50 decontaminated tomonts were added to confluent cultures of G1B cells, an established fish gill cell line (*12*). Each flask

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