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A constitutive gene expression system derived from the *tCUP* cryptic promoter elements

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Abstract A limited number of constitutive promoters have been used to direct transgene expression in plants and they are often derived from non-plant sources. Here, we describe novel gene-regulatory elements which are associated with a cryptic constitutive promoter from tobacco, *tCUP*, and modifications that were made to create a strong gene-expression system that is effective across all living cell types from a wide range of plant species, including several important crops (*Arabidopsis*, canola, flax, alfalfa, tobacco). The *tCUP* 5′ untranslated region was mutated to eliminate translational interference by upstream ATGs, and the influence of the Kozak consensus sequence on the levels of a β-glucuronidase (GUS) reporter gene activity was demonstrated. These modifications resulted in expression that was greatly enhanced in all organs. A TATA consensus sequence was added to the core promoter to complement an existing Initiator (Inr) sequence. Although this addition was known to elevate core promoter activity by 3-fold the additive effect on the overall gene-expression system was marginal in all of the transgenic plants tested. Two transcriptional enhancers were identified and the region containing them were oligomerized, yielding a significant increase in marker gene-expression in some but not all plant species. In general, the enhanced *tCUP* gene-expression system generated levels of GUS activity which exceeded that of the 35S promoter in most plant species and the elevation in activity occurred uniformly among the various plant organs. The potential benefit of cryptic elements for the construction of gene-expression systems for crop species is discussed

Keywords Constitutive promoter · Cryptic elements · Enhancer elements · Transgenic crop plants

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Dedicated to the memory of Tina Saffioti
K. Malik and K. Wu have contributed equally to the work and are both considered to be first authors

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Introduction

Constitutive promoters of plant genes have generally not been studied as extensively as promoters associated with tissue-specific and developmentally regulated genes. For the production of transgenic plants the cauliflower mosaic virus 35S promoter (Odell et al. 1985) and promoters of the *Agrobacterium* T-DNA genes (Bevan et al. 1983) are most frequently used. The compositions of these promoters are similar to those controlling plant genes in that they include a variety of enhancers and core promoter elements that may be interchangeable among each other and the promoters of plant genes. New combinations of these elements may have a dramatic effect on the activity of the composite promoters derived from them (Kay et al. 1987; Comai et al. 1990; Ni et al. 1995). Constitutive expression generated by the 35S promoter results from the combination of tissue-specific elements within it (Benfey et al. 1990a, b) and these may be recognized differently among plant species (Benfey and Chua 1990). Similar findings were discovered for the octopine synthase (Kononowicz et al. 1992) and nopaline synthase (An et al. 1988) promoters. A variety of plant constitu-

tive promoters have been isolated (Callis et al. 1990; Zhang et al. 1991; de Pater et al. 1992; Mandel et al. 1995; Baszczynski et al. 1997) but generally they have not been as extensively examined.

Constitutive promoter elements have been found in diverse genes including plant genes that are not constitutively expressed; for example, the promoters of the cytosolic glutamine synthetase gene of soybean (Tercé-Laforgue et al. 1999) and the pea *rbcS-3A* gene (Lam et al. 1990). Another kind of constitutive element associated with genes are the A/T-rich elements that can elevate expression of reporter genes without tissue-specificity (Sandhu et al. 1998). Quantitative enhancers of transcription, such as Q elements, also exist but they act in tissue-specific expression (Hamilton et al. 1998). It now seems likely that constitutive elements are important in the regulation and evolution of many plant genes; however, their mode of action in the combinatorial model of plant promoters is just beginning to be studied and they have not yet been exploited for generating synthetic and/or composite promoters for the expression of cloned genes in transgenic plants.

Another source of constitutive regulatory elements are cryptic gene-regulatory elements. These elements are silent at their native locations within the plant genome but can be activated by rearrangements that position them adjacent to genes in a configuration that promotes gene expression (Fobert et al. 1994; Cocherel et al. 1996; Okresz et al. 1998; Foster et al. 1999; Plesch et al. 2000; Salgueiro et al. 2000). They are believed to be abundant in the plant genome and can generate diverse expression profiles (Fobert et al. 1994). We have discovered a tobacco cryptic promoter, *tCUP*, by T-DNA tagging, that confers strong constitutive expression on a GUS (β-glucuronidase) marker gene in transgenic tobacco (Foster et al. 1999) and transgenic *Arabidopsis* (Wu et al. 2001). The promoter region contains constitutive enhancers, a TATA-less core promoter and a functional Initiator (Inr) element (Wu et al. 2001). The leader sequence contains a strong translational enhancer element (unpublished). The *tCUP* cryptic elements can be combined with elements from the 35S promoter to create active composite promoters showing that cryptic promoters have the capacity to play a role in the evolution of gene-expression patterns (Wu et al. 2001). In this study, we have modified the cryptic elements and their configurations within the *tCUP* sequence and have studied the effectiveness of these modifications on heterologous gene expression in a wide range of transgenic plants. This enhanced *tCUP* gene-expression system provides an effective alternative for the expression of heterologous genes in plants.

Materials and methods

Plasmid construction

Promoter deletion and replacement constructs were created in the vector pBI221 (Clontech), which contains the GUS (*uidA*) coding region driven by the CaMV 35S promoter and the nopaline synthase (*nos*) terminator. The CaMV 35S promoter of pBI221 was replaced with the deletion fragments of *tCUP* (GenBank accession number AF133844) to generate *-394tCUP-GUS-nos*, *-197tCUP-GUS-nos* and *-62tCUP-GUS-nos* as described in Wu et al. (2001). The numbers represent the nucleotide numbers relative to the transcription initiation site (Foster et al. 1999).

To generate *tCUP*Δ, the fragment from +24 to +134 relative to the transcription start site was amplified by PCR *Taq* polymerase using the first primer, with a *Bgl*II site (CCGCCACAGATCTATCCTCTTATCTCTCAAACTC) which mutated the ATG at the *Nde*I restriction site to an ATC. The second primer (TTGAGAAGCCATGGTGGCCGGTGGGTTTTGAGG) introduced a Kozak consensus sequence (CCACCATGG; Kozak 1984) and mutated four bases of the native *tCUP-GUS-nos* in-frame ATG start site AATACATGG. The PCR product was first digested with *Bgl*II and blunt-end filled using T4 DNA polymerase. The *tCUP* plasmid was digested with *Nde*I, blunt-end filled and ligated to the blunt-end filled PCR product. The PCR-*tCUP* ligation was digested a second time with *Nco*I and *Eco*RI and ligated to the *Nco*I and *Eco*RI *nos* fragment of pBI526 (Datla et al. 1993), generating an intermediate plasmid. The *GUS-nos Bam*HI to *Eco*RI fragment from pBI221 was then subcloned into the intermediate plasmid replacing *nos* and generated *tCUP*Δ*-GUS-nos*. The sequence integrity was verified by sequencing.

To generate *EntCUP1*, the promoter fragment from –394 to +115 relative to the transcription start site of *tCUP* (Foster et al. 1999) was amplified by PCR with *Taq* DNA polymerase, using the pr-1 (TTGCCTGCAAGGGATCTTCTG) and pr-2 (CGGGATCCTCTAGACCGGTGGGTTTTGAGGTGAG) primers. The PCR product was digested by *Pst*I and *Bam*HI, and the resulting fragment was used to replace the *Pst*I and *Bam*HI fragment in pBI221. To generate *EntCUP2*, the TATA consensus sequence (TATATAA) was used to substitute the sequence from –30 to –36 in the core promoter region of *EntCUP1* as described previously (Wu et al. 2001). To generate *EntCUP3*, the fragment from –394 to –62 was PCR-amplified by using the pr-1 and pr-3 (TCAAATGCATGGATCAAAAGGGGAAAC) primers. The PCR product was digested with *Pst*I and *Nsi*I, and two copies of the resulting fragment were subcloned into the *Pst*I site of *EntCUP2*. To generate *EntCUP4* and *EntCUP5*, a 300-bp coding region from the GUS ATG to the GUS *Sna*BI site was amplified by PCR *Taq* polymerase using the first primer (TCTAGAGGATCCACCATGGGTCCGTCCTGTAGAAACCCC), which introduced an *Xba*I/*Bam*HI/*Nco*I multicloning site with the Kozak consensus sequence at the GUS ATG, and a second primer (GUS antisense: CGGTGATACGTACACTTTTCC). The PCR product was digested with *Bam*HI and *Sna*BI. *EntCUP2* and *EntCUP3* were digested with *Bam*HI and *Sna*BI in order to remove the 300-bp GUS coding region and was ligated to the PCR product, generating *EntCUP4* and *EntCUP5*, respectively.

DNA sequence analysis

All PCR products were sequenced to make sure that mutations were not introduced into the sequences. Dye primer sequencing of plasmid DNA was performed using an automated sequencing system (Applied Biosystems). DNA sequence analysis was carried out using the DNASIS program (Hitachi Software Engineering Co., Ltd).

Plant transformation and selection

All of the promoters and GUS fusions were subcloned into the pCAMBIA2300 (Cambia, Canberra, Australia) binary vectors for plant transformation. Plant transformation plasmids were electroporated into *Agrobacterium tumefaciens* GV3101, as described by Shaw (1995), or AGL 1 (Rogers et al. 1986) by triparental mating. For particle bombardment the constructs were cloned into pTZ19R (Fermentas).

The *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* (ecotype Columbia) was performed according to Clough and Bent (1998) as modified by Wu et al. (2000b). Tobacco (*Nic-*

otiana tabacum, SR1) leaf-disc transformation was performed as described previously (Miki et al. 1993).

Canola (*Brassica napus*, cv Westar) hypocotyl transformation was performed by surface-sterilizing seeds in 70% Javex for 15 min, followed by three rinses in sterile distilled water. Seeds were germinated on half-strength MMO medium (Gibco, cat. 23118-078) pH 5.8, containing 1% sucrose solidified with 0.8% phytagar (Gibco cat. 10675-031) inside Magenta containers under 16 h daylength illumination (70–80 μE) at 25 °C for 4–5 days. *Agrobacterium* cultures were grown overnight in LB medium with appropriate antibiotics, then 1 ml of bacteria was added to 39 ml of bacterial base solution (MS medium salts with B_5 vitamins, 2% sucrose, 1 mg/l of 2,4-D, 1% DMSO). Fully-expanded cotyledons were cut at the base of the petiole and placed in bacterial base solution containing *Agrobacteria*. Approximately 500 cotyledons were incubated per 40-ml of bacterial base solution containing bacterial cells, in a 150 × 10 mm Petri dish at room temperature for 10 min. The bacteria and liquid was aspirated, the dishes were wrapped in parafilm and placed at 28 °C for 2 days in darkness. The plates were then moved to a 4 °C incubator for another 3–4 days of culture in darkness. The cotyledonary explants were moved to selection medium (MS salts/B_5 vitamins) solidified with 0.7% phytagar, containing 4 mg/l of BA, and timentin (300 μg/ml) and kanamycin (20 μg/ml), pH 5.8, in 100 × 25 mm Petri dishes. Explants were transferred to fresh selection plates at 1-week intervals. Green regenerated shoots were placed on rooting medium (MS salts/B_5 vitamins 3% sucrose, 4 mg/l of NAA, solidified with 0.7% phytagar) and rooted shoots were then transferred to peat pellets in trays in a growth cabinet at 25 °C.

For flax transformation, flax seed (cv Norlin) was surface-sterilized by immersing the seeds in 50% Javex (commercial bleach = 5.25% sodium hypochlorite) for 20 min, then triple-rinsing with sterile, distilled, de-ionized water. Seed was plated on MS media (Murashige and Skoog 1962) and placed in a dark cupboard to germinate. After 5 days the hypocotyl tissue (the region between the root and the cotyledons) was isolated and chopped into tiny pieces 1–2 mm in length. The chopped hypocotyl pieces were placed on plates of MS media with 1 mg/l of n6-benzyladenine (BA) and plates were put in a growth cabinet (24 °C) under light for a 3-day pre-culture prior to inoculation. For transformation, *A. tumefaciens* strain AGL1 (Rogers et al. 1986) was grown as an overnight culture by inoculating a small amount of a glycerol stock into liquid LB media plus appropriate antibiotics. Prior to transformation the culture was diluted with LB to a *D*(600 nm) of 0.4 to 0.6. The hypocotyl tissue was soaked in the *Agrobacterium* culture for 10 min, blotted on sterile filter paper to remove excess bacteria, then plated on MS+BA plates on top of sterile filter paper. These plates were returned to the tissue-culture cabinet under light and co-cultivation was allowed to proceed for 6 days. The tissue was then transferred to selection media (MS media with BA, 300 mg/l of timentin and 50 mg/l of kanamycin) and placed in the tissue-culture cabinet under light. Callus and shoots were allowed to regenerate, with tissue transferred to fresh selection plates every 3 weeks. As shoots appeared, each was tested for GUS expression by removing a single leaf and staining with X-gluc solution. Shoots whose leaves stained blue were retained. These shoots were placed in magenta jars containing MS media with 300 mg/l of timentin to prevent *Agrobacterium* grow-back, where they developed roots. Plants were then transferred to soil and grown to maturity.

Alfalfa (*Medicago sativa* L. cv Rangelander, clone N4) plants were propagated in vitro. Petioles were cut into about 0.8-cm fragments. The explants were pre-cultured on SH2K medium for 2 days. SH2K medium consisted of Schenk and Hildebrandt medium (1972), 4.5 μM of 2,4-D, 0.9 μM of kinetin, 25 mM of proline, 0.4 mM of thioproline, 50 mM of potassium and 1.5% sucrose. After 2-day pre-culture, the explants were infected with *Agrobacterium*. The infected explants were then cultured on SH2K medium for 2 days. After 2-day co-culture, explants were transferred to SH2K medium supplemented with 50 mg/l of kanamycin and 300 mg of timentin. The explants were transferred to fresh medium every 2–3 weeks. The explants with callus were then transferred to BOi2Y medium for embryo development. BOi2Y medium consisted of Blaydes medium (Bingham et al. 1975) modified to contain 3% sucrose, 0.2% yeast extract, 100 mg/l of myo-inositol, and 0.25% gelrite, plus 300 mg/l of timentin and 50 mg/l of kanamycin.

Elongated mature embryos with well-formed cotyledons were picked up and transferred to half-strength MSO supplemented with 100 km/l of kanamycin and 300 mg/l of timentin for germination. MSO medium contained MS ingredients (Murashige and Skoog 1962), 2% sucrose and 0.25% gelrite. Embryos germinated were transferred to half-strength MSO in Majenta boxes. Well-established plants were transferred into soil in the greenhouse.

Transient expression analysis

Wheat callus production was induced by placing 14–20 day old embryos of the variety Sumai 3, embryo side down on callus induction MS medium (Murashige and Skoog 1962) with 2,4-D at 1.5 mg/l (Weeks et al. 1993). When significant callus production was observed within 5 to 31 days these were crushed onto filter paper, four embryos per plate, and transferred to fresh media in preparation for bombardment.

DNA preparation and bombardment were as described by Brown et al. (1994). The tissue was placed at position 10 in the device (18 cm from the DNA loading screen) and was bombarded once at 100 psi. Tissue was incubated in a growth chamber overnight following bombardment. For GUS specific-activity analysis, tissue was collected and ground in liquid nitrogen and either stored at –80 °C or immediately extracted according to Jefferson (1987), as modified by Vitha et al. (1993).

Transient expression analysis in tobacco leaves was performed by particle bombardment as described by Wu et al. (2000a).

Northern-blot analysis

Total RNA was isolated from 100 to 200 mg of plant tissues using Tri Pure Reagent as described by the manufacturer (Boehringer Mannheim). Northern blots were prepared by electrophoresis of 5–10 μg samples of total RNA through agarose gels in the presence of formaldehyde, followed by transfer to nylon membranes. Northern blots were probed with ^{32}P-labelled probes. Pre-hybridization and hybridization were performed at 65 °C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS and 1 mM EDTA (Strommer et al. 1993). Filters were washed once for 15 min in 2 × SSC with 0.1% SDS at room temperature, then twice for 20 min in 0.1 × SSC, 0.1% SDS at 65 °C. The damp filters were autoradiographed at –80 °C using two intensifying screens. Filters were stripped in 5 mM of Tris-HCl, pH 7.5, 1 mM of EDTA and 0.05% SDS at 100 °C for 2 min, when re-probing was required.

GUS assays

To assay GUS activity of transgenic plants, the leaves from the T1 generation of transgenic plants were harvested and frozen in liquid nitrogen. Then 100–200 mg of leaf tissue were ground in extraction buffer consisting of 50 mM of sodium phosphate, pH 7.0, 10 mM of 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid, 0.1% Triton X-100, 10 mM of 2-mercaptoethanol and 0.1% sodium lauryl sarcosine. The extract was sonicated two times for 20 s in a bath-type sonicator, centrifuged for 15 min (4 °C), and the supernatant was collected for GUS analysis.

For GUS specific-activity measurements (Jefferson 1987), 50 μl of the crude extract from leaf tissues or protoplasts were incubated at 37 °C with 1 mM of 4-methylumbelliferyl glucuronide in 0.3 ml of GUS assay buffer [50 mM of $NaPO_4$, pH 7.0, 10 mM of EDTA, 0.1% (v/v) Triton X-100, 10 mM of β-mercaptoethanol]. After 0, 0.5, 1 and 2 h of incubation, 0.1-ml aliquots were removed and added to 1.9 ml of 0.2 M Na_2CO_3 to terminate the reaction. GUS activity was expressed as picomoles of 4-methylumbelliferone per mg of protein per min. Protein was measured using the BioRad Protein Assay kit based on the Bradford dye-binding procedure.

For GUS histochemical staining, tissue was incubated in a 0.5 mg/ml solution of 5-bromo-4-chloro-indolyl β-D-glucuronide in 100 mM of sodium phosphate buffer, pH 7.0, infiltrated in a vacuum for half an hour and incubated at 37 °C overnight. Following the incubation, tissue was washed in 70% ethanol to remove the chlorophyll.

For detection of GUS activity in cells, the pre-fixation and reaction with 5-bromo-4-chloro-indolyl β-D-glucuronide(x-gluc) were according to Jefferson et al. (1987). The tissues in the GUS-assay solution were infiltrated in a vacuum for half an hour and incubated at 37 °C overnight. Following the incubation, tissue was washed in 70% ethanol to remove the chlorophyll. The dehydration, slide preparation, embedding in paraffin, sectioning (20 μm), wax removal, and mounting were performed according to Li et al. (1996). Microphotography was under a Zeiss optical microscope.

Results

tCUP transcriptional enhancers

Previous studies on transgenic *Arabidopsis* (Wu et al. 2001) showed that a trancriptional enhancer responsible for the constitutive activity of *tCUP* was located in the proximal promoter region between positions –62 and –197 (Fig. 1A); however, the activity of the 5′ deletion at position –197 (*-197tCUP-GUS-nos*; Fig. 1B) was reduced relative to the 5′ deletion at position –394 (*-394tCUP-GUS-nos*; Fig. 1B). This was demonstrated in tobacco by transient expression analysis (Fig. 1C) and raised the possibility that other elements exist upstream of the constitutive enhancer. Transient expression analysis of the DNA fragment from –197 to –394 alone (*-394/197tCUP-GUS-nos*; Fig. 1B, C) revealed the presence of a second *tCUP* enhancer that was active in both forward and reverse (*-394/197RtCUP-GUS-nos*; Fig. 1B, C) orientations with respect to the minimal *-62tCUP* core promoter (Fig. 1B, C). Of particular interest are the A/T-rich sequences in this fragment (Fig. 1A) as they resemble sequences that have been shown to act as quantitative enhancers (Sandhu et al. 1998). An electrophoretic mobility shift assay using fragments spanning this region did not reveal the presence of a strongly binding protein in tobacco nuclear extracts under conditions that revealed binding to the cryptic constitutive enhancer (Wu et al., unpublished data).

Further experiments showed that the duplicated fragment from –62 to –394 [*2X(-394/62)-GUS-nos*; Fig. 2A], which carried both *tCUP* enhancer elements, could be combined with core promoters from plant genes. The promoter (-206LTP) from the wheat, aleurone-specific, lipid transfer protein gene is truncated at position –206 relative to the translational start codon and has 90 bp of

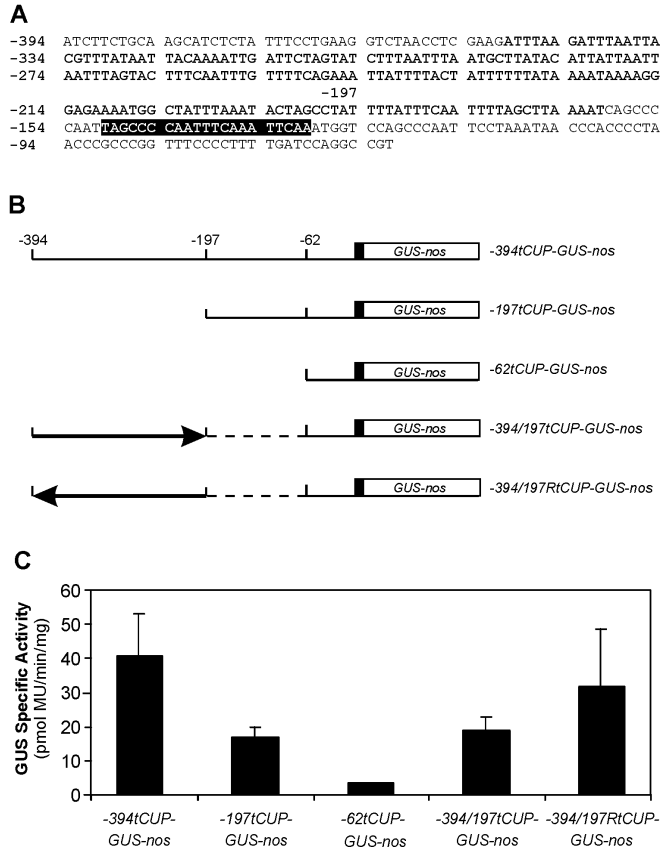

Fig. 1A–C Analysis of the *tCUP* transcriptional enhancer region in tobacco leaf by transient expression. **A** The sequence of the native *tCUP* fragment from position –394 to –62 relative to the transcription start site. The constitutive cryptic enhancer, *Cce*, within the –197/–62 fragment is *highlighted* and the A/T-rich tracts present in the –394/–197 fragment are shown in *bold*. **B** Maps of the *tCUP-GUS-nos* gene 5′ deletions at positions –394, –197 and –62 compared with constructs in which the –394/–197 fragment were fused to the minimal *-62tCUP* promoter in forward and reverse orientations. The *shaded box* represents the N-terminal fusion to GUS-nos. **C** Analysis of GUS specific activity in tobacco leaves (average ± SE, n = 12) generated by the constructs in **B** delivered by transient expression using particle bombardment

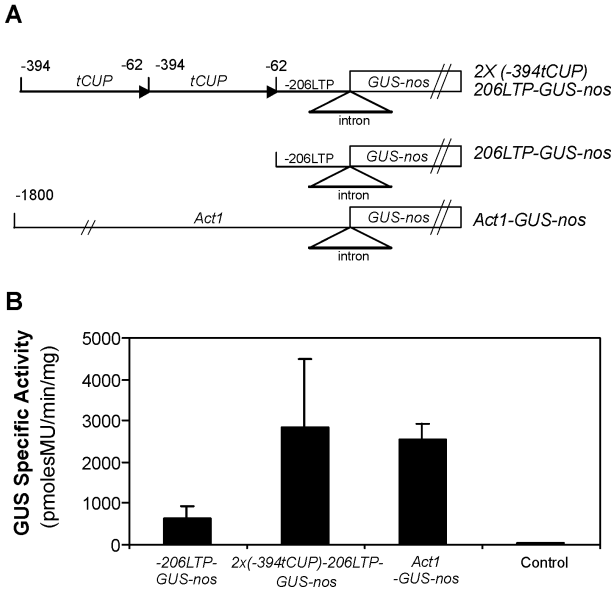

Fig. 2A, B Analysis of the duplicated *tCUP* enhancer region in wheat embryos by transient expression using particle bombardment. **A** The duplicated *tCUP* enhancer region [2X(–394/–62)]was fused to the -206LTP promoter from wheat and compared with the rice actin-1 promoter fused to GUS (*pAct 1D*; McElroy et al. 1990). All constructs contained the rice *Act 1* intron in the 5′ UTR. **B** Analysis of GUS specific activity (average ± SE, n = 15) in wheat embryo cultures following particle bombardment. The control was particle bombardment with vector DNA alone

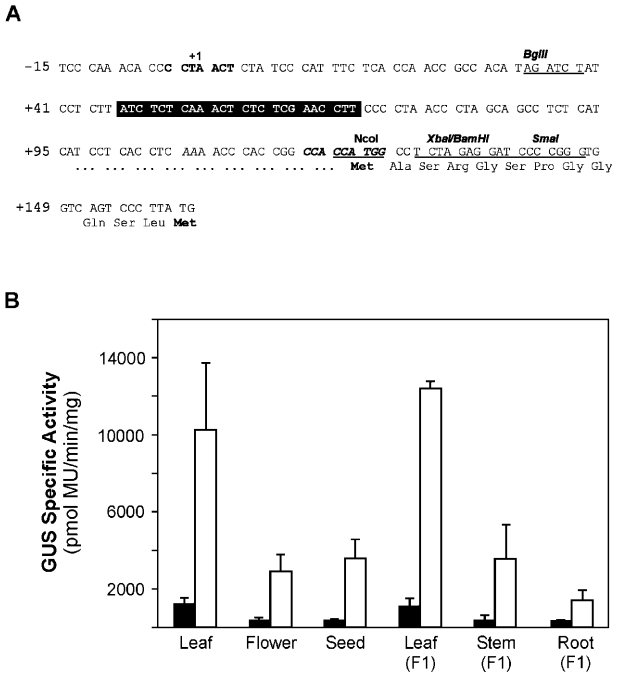

Fig. 3A, B Modification of the *tCUP* 5′ untranslated region to create *EntCUP* Δ. **A** The DNA sequences from the transcription start site to the GUS ATG are shown for *EntCUP* Δ. The upstream ATG in *tCUP* was eliminated by the creation of the *Bgl*II site. The translational enhancer element was not disturbed and is indicated by the *box*. The Kozak consensus sequence in shown in *italics*. The sequence of the N-terminal fusion to GUS is shown below the DNA sequence. The *Nco*I site that was used to facilitate cloning of other genes is *underlined* as are the other cloning sites. **B** Analysis of GUS specific activity (average ± SE, $n = 5$) in organs of transgenic tobacco transformed with the *tCUP-GUS-nos* (solid boxes) and *EntCUP* Δ-GUS-nos (open boxes) genes. Leaves, flowers and seeds were analysed for primary transformants, and leaves, stems and roots were analysed for progeny plants (*F1*)

promoter sequence upstream of the putative TATA box. With the tissue-specific elements deleted it is a very weak promoter (Fig. 2A, B); however, addition of the duplicated *tCUP* enhancer region substantially elevated the –206LTP promoter activity in wheat to levels that exceeded that of the rice actin promoter (Fig. 2A, B). In wheat, the *tCUP-GUS-nos* gene activity was very low and similar to that of the control (data not shown). The combined observations indicate that the *tCUP* enhancer elements have a broader range of activity, including monocots, than the *tCUP* promoter.

Modification of the mRNA leader sequence

The sequence of the gene fusion, *tCUP-GUS-nos*, combined with Western-blot analysis indicated that the original insertion of the T-DNA carrying the promoterless GUS-nos gene (Fobert et al. 1991) had created a translational fusion with the GUS gene (Foster et al. 1999; Fig. 1B). Furthermore, another upstream out-of-frame ATG was found in the 5′ untranslated region (Foster et al. 1999). To enhance the *tCUP* sequence for heterolo-

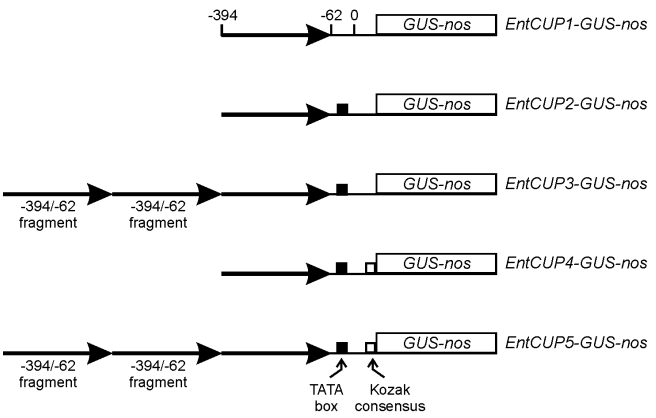

Fig. 4 Enhanced *tCUP* constructs. *EntCUP1-GUS-nos* contains the *tCUP* sequence truncated at position –394 and contains a single copy of the enhancer region (*arrow*). The 5′ untranslated region has been modified to eliminate the upstream ATG and the N-terminal fusion peptide. It does not possess the Kozak consensus sequence, as in *EntCUP Δ-GUS-nos*. *EntCUP2-GUS-nos* has the addition of a TATA consensus sequence to the core promoter region at position –30, relative the transcription start site, in addition to the modifications introduced into *EntCUP1-GUS-nos*. *EntCUP3-GUS-nos* has three direct repeats of the enhancer region (–394/–62) in addition to the modifications introduced into *EntCUP2-GUS-nos*. *EntCUP4-GUS-nos* is the same as *EntCUP2-GUS-nos* except that the Kozak consensus sequence has been introduced. *EntCUP5-GUS-nos* is the same as *EntCUP3-GUS-nos* except that the Kozak consensus sequence has been introduced

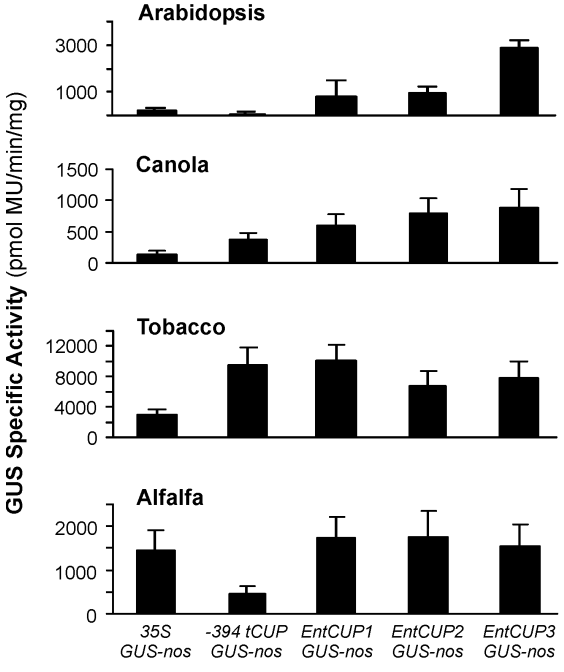

Fig. 5 Analysis of GUS specific activities (average ± SE) in leaves of independent transgenic *Arabidopsis* ($n = 16–21$), canola ($n = 16–20$), tobacco ($n = 24–25$) and alfalfa ($n = 15–23$) lines transformed with *-394tCUP-GUS-nos*, *EntCUP1-GUS-nos*, *EntCUP2-GUS-nos*, and *EntCUP3-GUS-nos*. For comparison, parallel experiments were performed with *35S-GUS-nos*

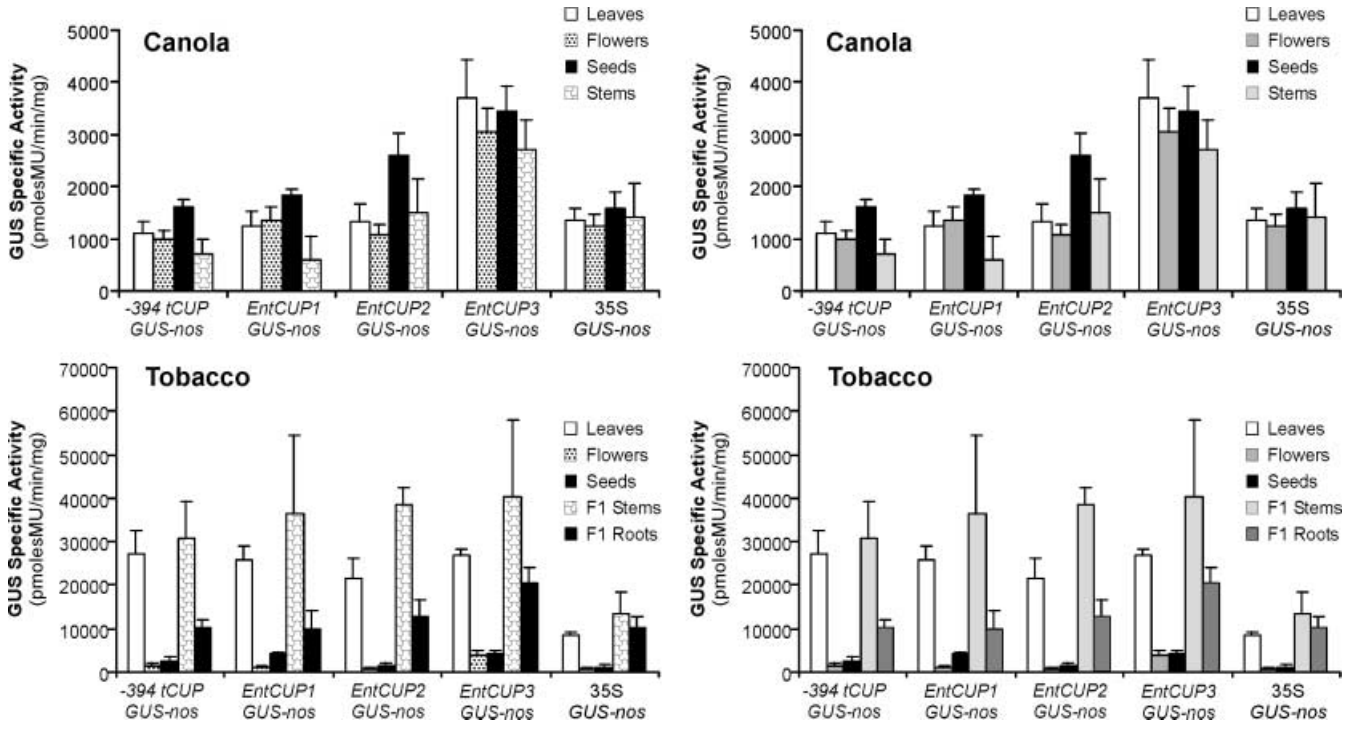

Fig. 6 Analysis of GUS specific activities (average ± SE) in leaves, stems, floral buds and immature seeds of individual transgenic canola plants described in Fig. 5 (n = 15), and leaves, flowers, seeds of primary regenerants and progeny leaves, stems and roots of individual tobacco plants described in Fig. 5 (n = 5)

gous gene expression, the leader sequence was modified by eliminating the upstream ATG at the *Nde*I site by mutating it to a *Bgl*II site (Fig. 3A). An *Nco*I site with a Kozak consensus sequence was also created at the ATG codon in the *tCUP* sequence to facilitate the cloning of other genes (Fig. 3A). The new sequence, *EntCUP*Δ, retained part of the N-terminal fusion (Fig. 3A); however, this peptide did not appear to affect GUS activity relative to native GUS (Foster et al. 1999). As shown in Fig. 3B, the *EntCUP*Δ*-GUS-nos* gene activity was enhanced by 5 to 11-fold over the native *tCUP-GUS-nos* gene fusion in all organs (leaf, stem, root, flower, seed) of transgenic tobacco.

The *tCUP* 5′ untranslated region (5′ UTR) also harbours a strong translational enhancer element that contributes significantly to the overall activity of *tCUP* (unpublished data). The sequence of the translational enhancer was not disturbed in *EntCUP*Δ (Fig. 3A).

Enhanced *tCUP* gene-expression system

A series of five constructs was generated (Fig. 4) to accumulate modifications to the *tCUP* sequence in a stepwise fashion. *EntCUP1* was truncated at position –394 as this fragment contained all of the upstream sequence essential for *tCUP* activity (Wu et al. 2001). The leader sequence was similar to *EntCUP*Δ in that upstream ATGs were eliminated. It differed from *EntCUP*Δ in that the sequence for the N-terminal peptide fusion to GUS was eliminated and the Kozak consensus sequence was removed so that a direct comparison with the *35S-GUS-nos* gene could be made. The 41-nucleotide leader sequence of the *35S-GUS-nos* gene did not contain upstream ATGs or a Kozak consensus sequence. *EntCUP2* was the same as *EntCUP1* except for the addition of the TATA consensus sequence to the core promoter region. Previous work showed that the TATA box increased the activity of the TATA-less *-62tCUP* core promoter by 3-fold (Wu et al. 2001). *EntCUP3* was the same as *EntCUP2* except that the upstream region (–394/–62), which contained both *tCUP* enhancers, was triplicated. *EntCUP4* and *EntCUP5* were the same as *EntCUP2* and *EntCUP3*, respectively, except that the Kozak consensus sequence was re-introduced to optimize expression.

To compare the activities of *EntCUP1-GUS-nos*, *EntCUP2-GUS-nos* and *EntCUP3-GUS-nos* the genes were inserted into *Arabidopsis*, canola, tobacco and alfalfa by *Agrobacterium*-mediated transformation. In *Arabidopsis* and canola, *EntCUP1-GUS-nos*, *EntCUP2-GUS-nos* and *EntCUP3-GUS-nos* generated progressively higher levels of mean GUS activity compared with *-394tCUP-GUS-nos* and *35S-GUS-nos*, indicating that the modifications to the 5′ UTR, core promoter and promoter enhancer regions were additive (Fig. 5). Analysis of canola leaf, stem, floral buds and immature seeds showed that the enhancements were not organ-specific but occurred non-specifically throughout the plant (Fig. 6). Northern-blot analysis of individual plants showed that the level of GUS mRNA generated by the *35S-GUS-nos* was generally higher than those for the *tCUP* constructs, illustrating the importance of the translational enhancer to the overall activity of *tCUP*. Oligomerization of the *tCUP* enhancer region in *EntCUP3-GUS-nos* elevated the

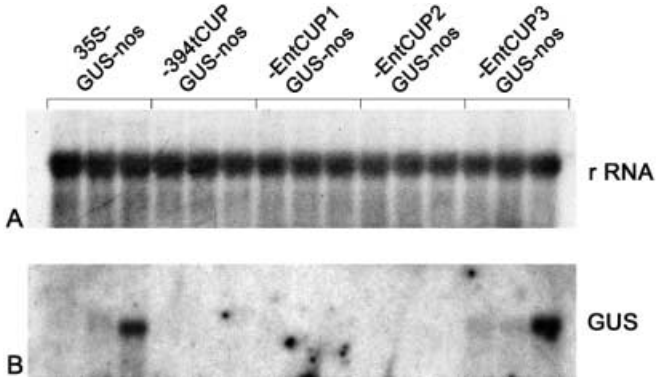

Fig. 7 Northern-blot analysis showing the relative GUS mRNA levels in leaves of individual transgenic canola plants transformed with *35S-GUS-nos*, *-394tCUP-GUS-nos*, *EntCUP1-GUS-nos*, *EntCUP2-GUS-nos* and *EntCUP3-GUS-nos*. As a control for equal loading of the wells, hybridization of the same filter with an rDNA probe pTA71 (Gerlach and Bedbrook 1979) is shown

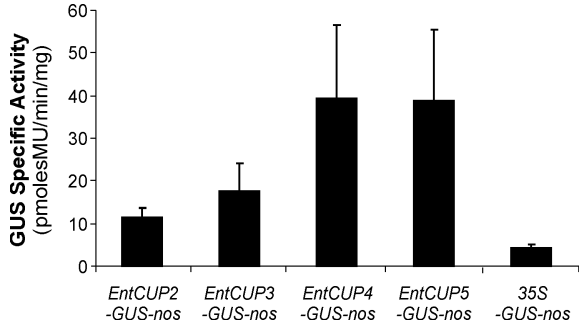

Fig. 8 Analysis of GUS specific activity in tobacco leaves (average ± SE, n = 12) generated by the constructs illustrated in Fig. 4 by transient expression using particle bombardment

mRNA levels to that seen for the *35S-GUS-nos* gene (Fig. 7)

In tobacco, the levels of GUS expression were basically the same for each of the *tCUP* constructs (Fig. 5). In this species, the level of *-394tCUP-GUS-nos* expression prior to enhancement was already very strong relative to the *35S-GUS-nos* gene (Fig. 5). Examination of the leaves, stems, roots, flowers and seeds revealed similar patterns of GUS activity for each of the constructs (Fig. 6). A similar situation was found in transgenic alfalfa except that the modification of the leader sequence in *EntCUP1-GUS-nos* greatly improved GUS expression levels (Fig. 5).

EntCUP4 and *EntCUP5* were examined by transient expression analysis in tobacco to determine if the Kozak consensus sequence, which had been removed, would enhance the activity of *EntCUP2* and *EntCUP3* further. As shown in Fig. 8, a significant increase (2–4-fold) in the overall expression of GUS was achieved.

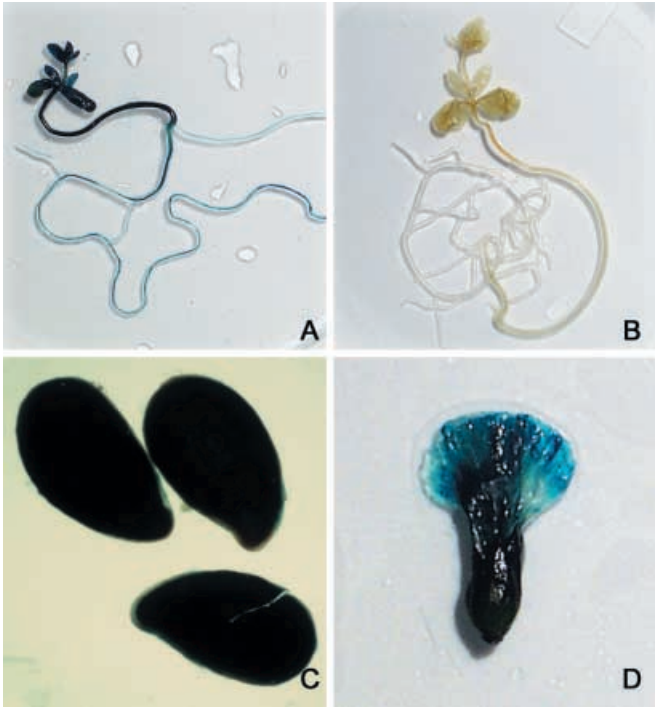

Fig. 9 Staining of GUS activity in seedlings (**A**), immature seeds (**C**) and flowers (**D**) of transgenic flax transformed with *EntCUP Δ-GUS-nos* truncated at –394 compared with seedlings of untransformed flax (**B**)

Constitutive expression in diverse plant species

The enhanced *tCUP* constructs *EntCUPΔ-GUS-nos*, *EntCUP1-GUS-nos*, *EntCUP2-GUS-nos* or *EntCUP3-GUS-nos* yielded expression in all organs of the dicot plant species examined. Histochemical analysis of GUS activity in seedlings, flowers and seeds of transgenic flax transformed with *EntCUP-GUS-nos* is illustrated in Fig. 9.

For a more-detailed analysis at the cellular level, cross sections of canola and tobacco plants transformed with *EntCUP3* were examined histochemically. GUS staining was observed in all living cell types of leaf, stem, root, flower and seeds, although with variation in staining intensity among the tissue and cell types (Fig. 10). The variation appeared to be associated with differences in cytoplasmic density among the tissues. The data indicated clearly that the enhanced *tCUP* constructs generated constitutive activity among diverse plants.

Discussion

This study demonstrated that *tCUP* is composed of many cryptic gene regulatory elements that are very useful for the expression of cloned genes in a wide range of agricultural crop species. *tCUP* is also very active in a wide range of vegetable, fruit tree and gymnosperm species (unpublished data). The levels of GUS activity achieved

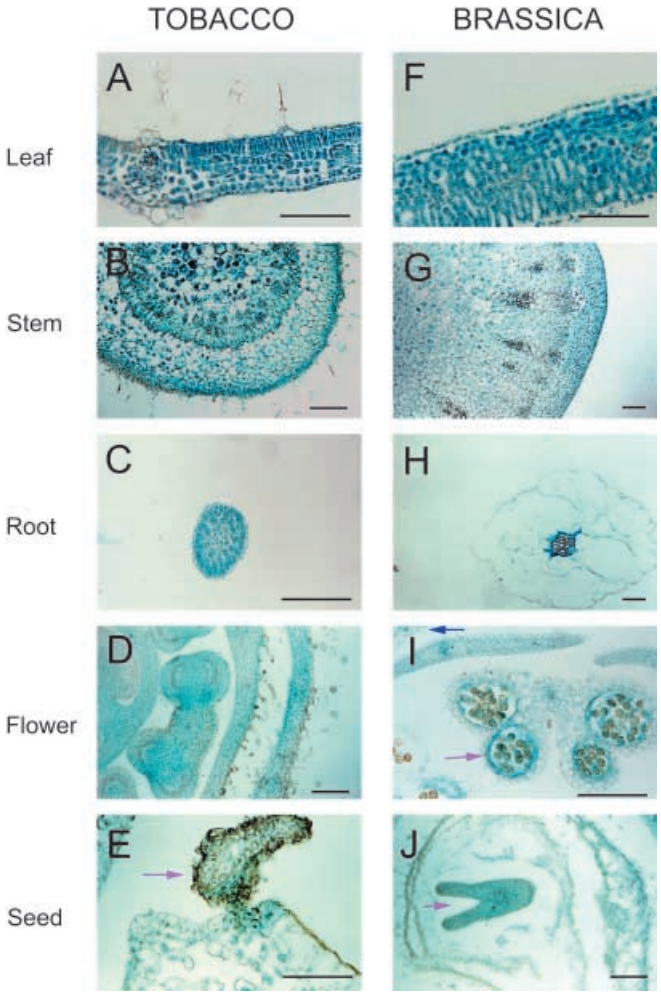

Fig. 10A–J Constitutive expression of GUS activity in cells of transgenic tobacco and canola (*Brassica*) transformed with *EntCUP3-GUS-nos*. **A**, **B**, **C**, **D** and **E** are tobacco sections; **F**, **G**, **H**, **I** and **J** are canola sections. **A** and **F** are leaf sections; **B** and **G** are from stems; **C** and **H** are from roots; **D** and **I** are from flowers; and **E** and **J** are from seeds. **C** is a cross section of the root meristem. **H** is a cross section of the elongated area of the canola root. The *pink arrow* in **I** indicates anthers with strong staining in the tapetum cells. The *blue arrow* in the **I** indicates the sepal. The *arrow* in **F** indicates immature seeds and in **J** indicates the meristem area of the heart-shape embryo. The embryo was surrounded by endosperm in degradation. GUS activity was observed in all the living tissues or cell types examined in both species. *Bars* represent 0.2 mm in length

were higher than that generated by the 35S promoter in all of the dicot plants examined. In some species, such as soybean, where unstable insertions were generated by particle bombardment-mediated transformation, the level of *tCUP* activity was lower than that obtained with the 35S promoter (unpublished data). Although *tCUP* is only active at very low levels in cereals, the individual elements may have broader activity among organisms. For instance, the *tCUP* transcriptional enhancers can be combined with other promoters such as the wheat-206LTP promoter thereby elevating activity to levels that are useful in cereals. The LTP promoter is aleurone-specific but the 5′ deletions which eliminated the tissue-specific enhancers yielded only weak constitutive-promoter activity. Moreover, the *tCUP* translational enhancer which exists in the 5′ untranslated region is active in all plant species, including monocots, dicots or gymnosperms and a wide range of other organisms including yeasts (unpublished data). The level of GUS mRNA generated by *tCUP* was lower than that generated by the 35S promoter indicating that the translational enhancer was important for the overall activity of *tCUP*; however, oligomerization of the*tCUP* enhancer region elevated the mRNA levels to those generated by the 35S promoter. Clearly, the combination of elements within *tCUP* is vital for the high levels of constitutive transgene expression that it can generate in plants.

Experiments were designed to alter the individual *tCUP* components and to examine the effects on the overall activity of *tCUP* in the organs and cells of different plants. The elimination of the upstream ATG in the 5′ untranslated region of *EntCUP1* elevated levels of GUS expression in most of the transgenic plants. The addition of the Kozak consensus also elevated activity significantly in transient expression assays. Although previous studies with transgenic *Arabidopsis* indicated that the addition of a TATA consensus to the *tCUP* core promoter increased core promoter activity by complementing the existing initiator sequence (Wu et al. 2001), the overall additive effect on *EntCUP2* was minor.

A strong constitutive cryptic enhancer, *Cce*, has been located in the proximal upstream region of *tCUP* (Wu et al., unpublished data). Further upstream is an A/T-rich region that is similar to sequences found in other plant promoters (Pedersen et al. 1989) where they may act as quantitative enhancer elements (Sandhu et al. 1998) or matrix attachment regions (Breyne et al. 1994). Partial deletion of this upstream region from *tCUP* has been correlated with a reduction in the overall expression of GUS levels in transgenic *Arabidopsis* and tobacco (Wu et al. 2001). Functional analysis of the region revealed it to be a second *tCUP* enhancer. Oligomerization of the region containing both transcriptional enhancers elevated the activity of *EntCUP3* in transgenic *Arabidopsis* and canola but not in transgenic tobacco and alfalfa. The combined modifications to the *tCUP* sequence varied in effectiveness among the plant species but did not alter the pattern of constitutive expression among the organs or at the cellular level.

The enhanced *tCUP* constructs described here are well-suited for regulating the constitutive expression of cloned genes for crop improvement and biotechnology. They have already been used effectively for modifying the MAPK signal-transduction pathways in transgenic tomato for stress resistance (Xing et al. 2001), for the functional analysis of histone de-acetylase genes, RPD3 and HD2, in transgenic *Arabidopsis* by expression of antisense constructs (Wu et al. 2000a, b) and for producing human interleukin 10 in transgenic tobacco (Menassa et al. 1999).

Recently, it has been found that the expression of transgenes driven by the 35S promoter may be unstable

in *Brassica* species under field conditions because of naturally occuring CaMV infections and homology dependent gene silencing resulting from their interactions (Al-Kaff et al. 2000). Cryptic gene-regulatory elements provide an interesting alternative to promoters associated with genes or viruses in that cryptic elements are not associated with any known physiological functions, and therefore silencing would not be expected to have any deleterious consequences to plants. Experiments are now underway to further enhance the levels of *tCUP*-mediated gene expression and to develop methods for the controlled silencing of the promoter in transgenic plants.

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