Transgenic Res (2010) 19:667–674
DOI 10.1007/s11248-009-9332-6

BRIEF COMMUNICATION

The 5′ untranslated region of the *VR-ACS1* mRNA acts as a strong translational enhancer in plants

Willem Wever · Emily J. McCallum · David Chakravorty · Christopher I. Cazzonelli · José R. Botella

Received: 1 July 2009 / Accepted: 25 September 2009 / Published online: 9 October 2009

Abstract The structure and function of untranslated mRNA leader sequences and their role in controlling gene expression remains poorly understood. Previous research has suggested that the 5′ untranslated region (5′UTR) of the *Vigna radiata* aminocyclopropane-1-carboxylate synthase synthase (*VR-ACS1)* gene may function as a translational enhancer in plants. To test such hypothesis we compared the translation enhancing properties of three different 5′UTRs; those from the *VR-ACS1*, the chlorophyll a/b binding gene from petunia (*Cab22L*; a known translational enhancer) and the *Vigna radiata* pectinacetylesterase gene (*PAE*; used as control). Identical constructs in which the coding region of the β-glucuronidase (GUS) gene was fused to each of the three 5′UTRs and placed under the control of the cauliflower mosaic virus 35S promoter were prepared. Transient expression assays in tobacco cell cultures and mung bean leaves showed that the *VR-ACS1* and *Cab22L* 5′UTRs directed higher levels of GUS activity than the *PAE* 5′UTR. Analysis of transgenic *Arabidopsis thaliana* seedlings, as well as different tissues from mature plants, confirmed that while transcript levels were equivalent for all constructs, the 5′UTRs from the *VR-ACS1* and *Cab22L* genes can increase GUS activity twofold to fivefold compared to the *PAE* 5′UTR, therefore confirming the translational enhancing properties of the *VR-ACS1* 5′UTR.

Keywords Translation efficiency · Promoter · Translational enhancer · Untranslated region

Abbreviations

Cab22L	Chlorophyll a/b binding
CaMV	Cauliflower mosaic virus
GUS	β-Glucuronidase
MUG	4-Methylumbelliferyl-β-D-glucuronide
PAE	Pectinacetylesterase
UTR	Untranslated region
VR-ACS1	*Vigna radiata* aminocyclopropane-1-carboxylate synthase

W. Wever · E. J. McCallum · D. Chakravorty · C. I. Cazzonelli · J. R. Botella (✉)
Plant Genetic Engineering Laboratory, School of Biological Sciences, University of Queensland, Brisbane, Queensland 4072, Australia
e-mail: j.botella@uq.edu.au

Present Address:
C. I. Cazzonelli
Australian Research Council Centre of Excellence in Plant Energy Biology, Research School of Biology, ANU College of Medicine, Biology and Environment, The Australian National University, Canberra, ACT 0200, Australia

Introduction

Due to their extraordinary importance, the mechanisms controlling gene expression in plants have been the focus of intense research over the past 20 years. Gene expression can be regulated by transcriptional,

post-transcriptional and post-translational processes but most of the research to date has focussed on understanding transcriptional control mechanisms through characterization of native plant promoters (Benfey and Chua 1990; Cazzonelli et al. 2005; Wu et al. 2003) and engineering of synthetic transcriptional enhancer domains (Cazzonelli and Velten 2008; Venter 2007). Post-transcriptional regulatory mechanisms such as gene silencing (Baulcombe 2004) and mRNA stability (Narsai et al. 2007) have been intensely studied over the last decade; however the role of the 5′UTR in controlling translation efficiency has received relatively little attention in plants.

Of the three fundamental steps of protein synthesis (initiation, elongation and termination), it has been proposed that the initiation step is the most important control point, and is usually considered the rate-limiting step of translation (Lodish 1976). While the 3′ untranslated and coding regions have the potential to influence translation (Lodish 1976), it is logical to correlate translational efficiency with the physical characteristics of the 5′UTR of the mRNA, where initiation of protein synthesis occurs.

The degree of secondary structure in the mRNA 5′UTR is a significant determinant of the rate of translation, which is thought to be dependent on the stability and location of such structure (Klaff et al. 1996). A high AU (or low GC) content, which reduces secondary structure formation, permits better ribosome scanning of the AUG start codon and consequently increased translation efficiency (Joshi et al. 1997). DNA microarrays of mRNAs contained in polysomal complexes from *Arabidopsis thaliana* have shown that genes with high GC content in their 5′UTR are generally poorly translated (Kawaguchi and Bailey-Serres 2005).

The length of the 5′UTR has been shown to affect translation efficiency in plants. Extremely short (<25 nt) leaders may impair translation fidelity, and in some cases cause the scanning 40S ribosomal subunit to skip over the first AUG codon, whereas longer (>175 nt) leader sequences may inhibit ribosome loading, perhaps due to increased secondary structure. In general lengths between 50 and 75 nt have been shown to promote optimal ribosome loading (Futterer and Hohn 1996; Kawaguchi and Bailey-Serres 2005). The presence of upstream open reading frames is known to significantly slow ribosome scanning and impair translation, while AUG sequence context (especially the presence of purines at positions −3 and +4) is associated with increased ribosome loading and therefore increased translation efficiency (Joshi et al. 1997; Kawaguchi and Bailey-Serres 2005).

Studies addressing the translational regulation properties of 5′UTRs in plants have been largely focused on viral leader sequences (Gallie 1993; Gallie and Walbot 1992; Turner et al. 1999). Some 5′UTRs of plant origin have been shown to influence translation efficiency such as the 66 bp chlorophyll a/b binding gene leader sequence from petunia (*Cab22L*), which was found to enhance translation by eightfold compared to a 31 nucleotide random leader sequence (Danthinne and van Emmelo 1990). This increase in translation was comparable to the 72 bp tobacco mosaic virus (TMV) Ω leader sequence, which enhanced translation by 12-fold (Danthinne and van Emmelo 1990). In a later study, De Loose et al. (1995) reported that the *Cab22L* 5′UTR enhances translation of *β*-glucuronidase (GUS) transcripts in tobacco calli, stably transformed tobacco leaves and their progeny as efficiently as the TMV Ω leader. The *Cab22L* 5'UTR has been utilized as a translation enhancer in a number of genetic studies (Brummell et al. 2003; Burgess et al. 2002; Fornara et al. 2004; Matsui et al. 2006; O'Keefe et al. 1994).

The identification and characterization of translation enhancing 5′UTRs derived from plant sources can prove very useful in the production of genetically modified (GM) plants. From an energetics perspective, increased translation efficiency is beneficial to plants as transcription and nuclear export of mRNAs are energy consuming processes. Additionally, the use of plant-derived instead of viral 5′UTRs in the production of GM plants may be perceived as more suitable by regulatory agencies and enhance public acceptance of such technologies. For example, the *Cab22L* 5′UTR is found in a number of genetically modified crops approved for human consumption in several countries (Centre for Biosafety Assessment, Technology and Sustainability, http://www.bats.ch/gmo-watch/GVO-report140703.pdf).

We have previously described the isolation and characterization of the aminocyclopropane-1-carboxylate synthase (*VR-ACS1*) promoter from *Vigna radiata* L. (mung bean) (Cazzonelli et al. 2005). In its original host, the *VR-ACS1* gene is strongly

induced by a number of stimuli including auxin, wounding and dehydration stress, but shows very little transcriptional activity under normal physiological conditions. Genetically modified *Nicotiana tabacum* (tobacco) and *Arabidopsis thaliana* plants containing the *VR-ACS1* promoter fused to two different reporter genes showed 4–6 times higher reporter activity than plants containing the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Cazzonelli et al. 2005). However, it was observed that *VR-ACS1* and CaMV 35S-driven transcript levels were similar, leading to the hypothesis that the *VR-ACS1* 5′UTR could act as a translational enhancer in plants (Cazzonelli et al. 2005). In this report we show that the *VR-ACS1* 5′UTR functions as a strong translational enhancer in plants.

Results and discussion

In order to determine the relative translational efficiency of the *VR-ACS1* 5′UTR we compared it with the 5′ leader regions of two other genes, the *Vigna radiata* pectinacetylesterase (*PAE*) (GenBank accession no. ×99348) (Breton et al. 1996) and the *Petunia* × *hybrida* (Mitchell) chlorophyll a/b binding protein (*Cab22L*) (Dunsmuir 1985). The *Cab22L* 5′UTR was chosen due to its proven strong translation enhancing qualities in transgenic plants (Danthinne and van Emmelo 1990; Harpster et al. 1988) while the *PAE* 5′UTR was chosen as a negative control since (a) it comes from the same species as *VR-ACS1* and (b) bioinformatics analyses (Fig. 1a) suggest that it is not expected to increase translational efficiency. The *Cab22L*, *VR-ACS1* and *PAE* 5′UTRs are 66, 87 and 96 bp long, respectively. In order to avoid any size effects between the *VR-ACS1* and *PAE* 5′UTRs, the later was trimmed to exactly 87 bp by excluding the first 9 nucleotides from its 5′ end. The *Cab22L* 5′UTR falls within the 50–75 bp leader sequence length for optimal ribosome loading (Futterer and Hohn 1996; Kawaguchi and Bailey-Serres 2005), while the *VR-ACS1* and *PAE* 5′UTRs are slightly larger than ideal.

Care was taken in designing test constructs to ensure they were identical except for the three 5′UTRs being studied. A β-glucuronidase gene containing an intron sequence from the castor bean catalase gene (Ohta et al. 1990) was chosen as reporter gene (iGUS). The presence of an intron ensures that any protein activity

(a)

5'UTR	Length	AU content	Minimum free energy (ΔG)
VR-ACS1	66 bp	62%	0.0 kcal/mol
Cab22L	87 bp	67%	-2.8 kcal/mol
PAE	87 bp	60%	-9.7 kcal/mol

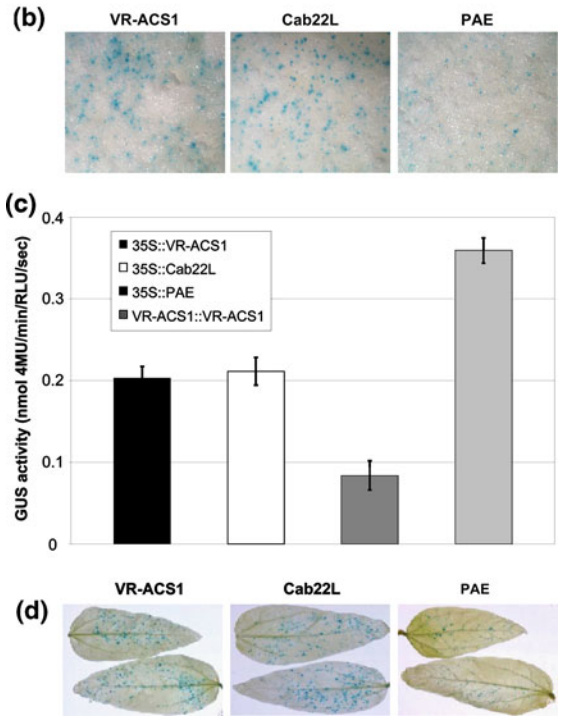

Fig. 1 The *VR-ACS1* 5′UTR behaves as a translational enhancer in tobacco and mung bean transient expression systems. **a** Comparison of 5′UTR sequence features. **b** Histochemical GUS assays in tobacco cell cultures. **c** Quantitative assay of GUS activity in tobacco cell cultures co-bombarded with test (pGTVm:: 5′UTR::iGUS) and normalization (p35sLuNt) vectors. GUS and LUC activities are referred to the amount of soluble protein and luciferase used to normalize the results (nmoles 4MU/min/RLU/sec). Error bars are ±SE of three independent particle bombardments measured in duplicate. **d** Histochemical GUS assays in mung bean leaves. All histochemical GUS assays were performed in duplicate revealing similar results

measured in transient assays can be solely attributed to expression of the reporter gene in plant cells, which unlike *Agrobacterium*, are capable of processing the intron to allow translation of the mature GUS transcript. All constructs contained the iGUS coding region under the control of the CaMV 35S promoter (Odell et al. 1985). Each of the 5'UTRs were fused upstream of the iGUS gene without adding any additional nucleotides as a result of the cloning strategy.

The AU content of the *PAE* 5′UTR is slightly lower than the *VR-ACS1* and *Cab22L* 5′UTRs (Fig. 1a). High AU content, also referred to as low GC content (Kawaguchi and Bailey-Serres 2005), is associated with reduced secondary structure, favourable ribosome scanning and increased translation efficiency (Joshi et al. 1997). The *Cab22L* 5′UTR, which is known to enhance translational efficiency, is predicted to form one small and one large hairpin loop with a minimum change in Gibbs free energy (ΔG) value of −2.80 kcal/mol (RNAfold WebServer, http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). Interestingly, the *VR-ACS1* 5′UTR is not expected to contain any secondary structure, and the *PAE* 5′UTR is predicted to form a complex and stable hairpin loop structure with a ΔG value of −9.70 (Fig. 1a). The absence of secondary structure could be indicative of high translational efficiency, as the tobacco mosaic virus (TMV) Ω leader sequence lacks any secondary structure and is known to act as an effective translational enhancer (Zaccomer et al. 1995).

The Kozak sequence, which plays a major role in translation initation and efficiency (Kozak 1989), is conserved in both the *Cab22L* and *PAE* 5′UTRs. For optimal ribosomal recognition of the AUG start codon, an A or G is present 3 nucleotides upstream of the start codon, which is followed immediately by a G nucleotide (Kozak 1989). All constructs in this study contain the consensus G downstream of the start codon as part of the iGUS coding sequence, however, the *VR-ACS1* 5′UTR contains a non-consensus C at the −3 position. Additionally, CAA trinucleotides are found in the *Cab22L* and *VR-ACS1* 5′UTRs, and may play a role in translation efficiency; a series of CAA motifs present in the TMV Ω leader sequence have been shown to recruit *trans*-acting factors (Wells et al. 1998) and enhance translation in plants (Gallie and Walbot 1992; De Amicis et al. 2007).

The relative strengths of the three 5′UTRs were studied by transient expression assays in tobacco cell cultures. Cell cultures were subjected to particle bombardment with each of the three constructs described above, followed by histochemical GUS staining. Our results repeatedly showed that cultures bombarded with the *VR-ACS1* and *Cab22L* 5′UTRs exhibited a similar number and intensity of GUS staining loci, while those bombarded with the *PAE* 5′UTR displayed fewer loci and lower intensity of GUS staining (Fig. 1b). These studies were confirmed by more comprehensive quantitative 4-methylumbelliferyl-*β*-D-glucuronide (MUG) assays of transient GUS activity. In order to compare GUS activity levels between independent bombardments, tobacco cell cultures were co-bombarded with a second construct (p35SLuNt) containing the luciferase reporter gene under the control of the CaMV 35S promoter (Cazzonelli et al. 2005). In this way, *β*-glucuronidase activity was normalized against luciferase activity to account for any differences in efficiency for each bombardment experiment. Quantitative MUG assay results correlated closely with the GUS histochemical observations. The *VR-ACS1* and *Cab22L* 5′UTR constructs directed similar levels of GUS activity, approximately 2 fold higher than the *PAE* 5'UTR construct (Fig. 1c). One of the drawbacks of transient assays is that it was not possible to determine whether transcription levels were identical for all three constructs. However, since the same promoter and transcription start site was used in all constructs, it is reasonable to assume that they resulted in equivalent transcriptional activity. Therefore, and given that the *Cab22L* 5′UTR functions as a strong translational enhancer in plants (Danthinne and van Emmelo 1990; Harpster et al. 1988), it is reasonable to suggest that the *VR-ACS1* 5′UTR also acts to enhance translation in a manner comparable to the *Cab22L* 5′UTR, while the *PAE* 5′UTR does not present obvious translation enhancing capabilities.

A binary construct containing approximately 2.4 kb of the *VR-ACS1* promoter and including the *VR-ACS1* 5′UTR fused to iGUS (Cazzonelli et al. 2005) was also tested in transient expression assays in tobacco cell cultures. GUS activity levels directed by this construct were approximately 2 fold higher than those observed in either the *VR-ACS1* or the *Cab22L* 5′UTR constructs, and more than 4 times higher than the *PAE* 5′UTR construct (Fig. 1c). These results agree with our previous study comparing the CaMV 35S and *VR-ACS1* promoters in transgenic tobacco and Arabidopsis lines (Cazzonelli et al. 2005).

Transient expression assays in mung bean leaf tissues using particle bombardment showed very similar results to those observed in tobacco cell cultures. Again, tissues bombarded with the *VR-ACS1* and *Cab22L* 5′UTR constructs exhibited similar numbers and intensity of GUS staining loci, while *PAE*–bombarded tissues displayed fewer and less

intense GUS stained loci (Fig. 1d). The similar results observed in tobacco and mung bean suggest that the translation enhancing properties of the *VR-ACS1* 5′UTR are not due to its use in heterologous species such as tobacco.

The transcriptional, translational and tissue-specific properties of the *VR-ACS1* 5′UTR were investigated in a number of stably transformed *Arabidopsis thaliana* lines. For this purpose, the CaMV 35S promoter:: 5′UTR::iGUS expression cassettes were cloned into a binary vector and used to produce genetically modified *Arabidopsis thaliana* lines. At least five independent transgenic lines were produced for each construct, and three of these lines containing a single T-DNA insertion according to segregation analysis were analysed.

Seeding, root, rosette and cauline leaf tissues were pooled from five individuals for each line and GUS activity measured in duplicate using quantitative MUG assays. Total RNA was extracted from the same tissue pools, and GUS transcript levels measured by northern analysis. All transgenic lines containing the *VR-ACS1* and *Cab22L* 5′UTRs consistently displayed twofold to fivefold higher GUS activity values than the *PAE* 5′UTR lines in all tissues and developmental stages examined (Fig. 2). These results are in agreement with the transient expression studies, where the *VR-ACS1* 5′UTR was shown to enhance GUS activity levels by 2 fold in tobacco cell cultures. Transcript levels for all tissues and transgenic lines analysed were very similar, proving that the differences in GUS activity observed were due to enhanced translation efficiency, and not transcriptional regulation (Fig. 2).

We conclude that the increased levels of GUS activity directed by the *VR-ACS1* and *Cab22L* 5′UTR constructs are the result of enhanced translational activity and not due to increased transcript levels. We do not expect that the longer length of the 87 bp *VR-ACS1* 5′UTR, compared to the 66 bp *Cab22L* 5′UTR is likely to contribute to the enhancer properties of the *VR-ACS1* 5′UTR, as the *PAE* 5′UTR is identical in length but consistently shows twofold fivefold lower activity in the various tissues tested. The fact that the *VR-ACS1* 5′UTR has shown translation enhancing properties in its own species, mung bean, as well as in unrelated species such as tobacco and Arabidopsis suggests that it can retain its properties in heterologous species. It still needs to be determined whether regulatory motifs are present within the *VR-ACS1* and *Cab22L* 5′UTR sequences that could play novel roles in recruiting factors that promote translation initiation or elongation. The translation enhancing properties of the *VR-ACS1* 5′UTR will be valuable for enhancing protein production in a range of biotechnological applications.

Materials and methods

Vigna radiata (mung bean) plants were grown under standard glasshouse conditions, and *Arabidopsis thaliana* grown under long day conditions (16 h photoperiod, 21°C). Tobacco cell cultures were grown as previously described (Cazzonelli et al. 2005).

Polymerase chain reaction (PCR) was performed using the Elongase proof-reading enzyme (Invitrogen) according to Chakravorty and Botella (2007). PCR products were cloned into pGEM-T Easy (Promega) or *Eco*RV digested pBlueScript II SK + (pBS). Restriction enzyme digestion and DNA sequencing confirmed the construction of intermediate and binary vectors, designed for transient and stable gene expression, respectively.

The pGTVa (Laurena et al. 2002) vector was used to construct vectors for transient expression. A 24 bp region between the CaMV 35S promoter and *Eco*RI restriction site was removed to ensure that each 5′UTR sequence was cloned immediately downstream of the CaMV 35S transcription initiation site. This was achieved by PCR amplification of the CaMV 35S promoter using T7-forward and 35S-reverse (CTCGAATTCTCTCCAAATGAAATG) primers, the product cloned into pBS, the promoter excised with *Xho*I/*Eco*RI and religated into pGTVa, producing the vector pGTVm.

The *VR-ACS1* 5′UTR::iGUS fusion was created by amplification of the iGUS reporter from pIG121 (Akama et al. 1992) using the forward primer, VRACS1-5UTR1 (TCAATTCCAATAAACTCAACACACTTTTTTACACTCCACACTCTAACCACATACACCATATGGATCCCTACAGGGTAAAT), containing 59 bp of the 3′ end of the *VR-ACS1* 5′UTR and 21 bp of the 5′ end of iGUS gene and a GUS reverse primer, GUS-3prime (TTA TCTAGATTAGGTAGCAATTCCCGAGGCTGTA) containing an *Xba*I site immediately downstream of the stop codon. This PCR fragment was cloned into

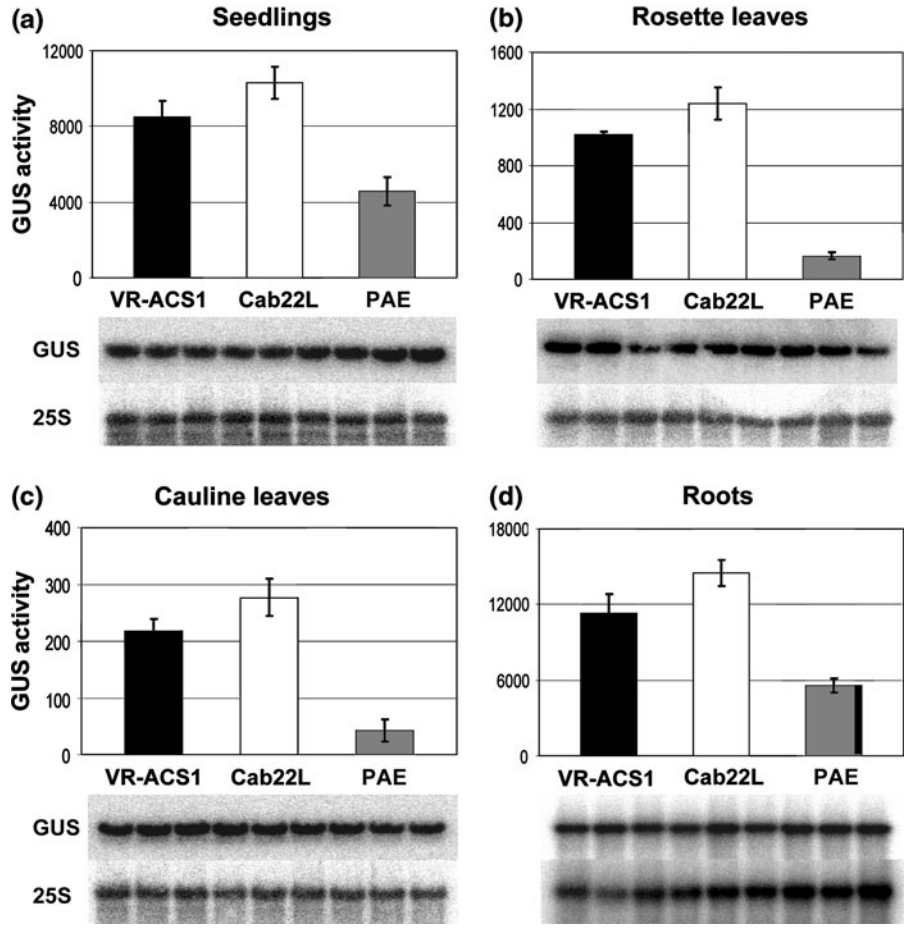

Fig. 2 The *VR-ACS1* 5′UTR enhances translation without affecting transcription. GUS activity and northern analysis of **a** 2 week old Arabidopsis seedlings, **b** mature rosette leaves, **c** cauline leaves and **d** root tissues. GUS activities are expressed in nmoles 4MU/min/mg of soluble protein. Error bars are ±SE of three independent lines; pooled tissue from five plants was measured in duplicate. Northern analyses show GUS transcription levels in Arabidopsis tissues for three independent lines. 25S ribosomal probe confirms equal loading of RNA samples

pBS and a second forward primer, VRACS1-5UTR2 (CCCGAATTCATCCTCTCTCCCACTTACTTCGATTTCATCAATTCCAATAAACTCAAC), containing an *Eco*RI site, a 20 bp overlap with the 3′ end of the 5′UTR already cloned and adding the remaining 28 bp to the 5′ end of the *VR-ACS1* 5′UTR sequence, was used again with the GUS-3prime primer to produce a PCR product containing the full length *VR-ACS1* 5′UTR (87 bp) fused with iGUS. The PCR product was cloned into pBS, subsequently excised with *Eco*RI/*Xba*I, and cloned into pGTVm; creating the vector pGTVm35S::*VR-ACS1* 5′UTR::iGUS.

For construction of the *Cab22L* and *PAE* 5′UTR iGUS fusions a similar strategy was utilized to amplify the 5′UTR-iGUS fusions. The *Cab22L* 5′UTR fusion was created in two steps. Firstly, the *Cab22L* 5′UTR was amplified with the forward primer Cab22L-5UTR1 (CTATTACTTCAGCAATAACAAAAGAACTCTTTTCTCTTCTTATTAAACCATGGATCCCTACAGGGTAAAT) and GUS-3prime reverse primer, and the product cloned into pGEM-T Easy. This plasmid was then used as template for a subsequent PCR, with the forward primer Cab22L-5UTR2–(CCCGAATTCGACTCGAGCTCATTTCTCTATTACTTCAGCAATAACA) and GUS-3′prime primer, and the product cloned into pGEM-T Easy. The *PAE* 5′UTR1 fusion was created in the same was as described for *Cab22L*, the PAE-5UTR1 (AGCTGAAGCATTTTACAGGCCACCATTTTCCTTGACTACCTTTCACTTACCATTTAAGAATGGATCCCTACAGGGTAAAT) forward primer used in the first PCR, and the PAE-5UTR2 (CCCGAATTCTCTTCCTCTGTGAAGACTTGTCGTTAGCTGAAGCATTTTACAGGC) forward primer in the second PCR step. The resulting 5′UTR::iGUS fusions were excised from pGEM-T Easy with *Eco*RI/*Xba*I and ligated into pGTVm35S::VR-ACS1 5′UTR::iGUS (replacing the *VR-ACS1* 5′UTR

and iGUS gene); thus creating the vectors pGTVm 35S::Cab22L 5′UTR::iGUS and pGTVm35S::PAE 5′UTR::iGUS.

Binary vectors for stable transformation of *Arabidopsis thaliana* cv. Columbia were created by excision of each 35S:: 5′UTR::iGUS expression cassette from pGTVm with *Hind*III/*Xba*I, and cloned into the binary vector pSLJ75515 (Mylne and Botella 1998).

pGTVm35S:: 5′UTR::iGUS vectors and a normalization vector, p35SLuNT (Cazzonelli et al. 2005) were used for transient analysis of gene expression. Particle bombardment of *Nicotiana tabacum* (tobacco) cell cultures and *Vigna radiata* (mung bean) leaves was performed as described by Cazzonelli et al. (2005), and stable transformation of *Arabidopsis thaliana* as described by Trusov et al. (2009). Transgenic Arabidopsis lines were selected by Basta resistance, and GUS histochemical staining of cauline leaf tissues of more than ten individual T1 transgenic lines was performed to establish a consensus staining pattern, from which three representative lines for each construct were selected. Single copy, homozygous lines were identified by segregation analysis using Basta.

Northern analyses were performed according to Purnell and Botella (2007) histochemical β-glucuronidase (GUS) assays were performed as described by Trusov et al. (2008); 4-methylumbelliferyl-β-D-glucuronide (MUG) assays and luciferase (LUC) assays were performed as described by Cazzonelli et al. (2005).

References

Akama K, Shiraishi H, Ohta S, Nakamura K, Okada K, Shimura Y (1992) Efficient transformation of *Arabidopsis thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. Plant Cell Rep 12:7–11

Baulcombe D (2004) RNA silencing in plants. Nature 431: 356–363

Benfey PN, Chua NH (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250:959–966

Breton C, Bordenave M, Richard L, Pernollet JC, Huet JC, Perez S, Goldberg R (1996) PCR cloning and expression analysis of a cDNA encoding a pectinacetylesterase from *Vigna radiata* L. FEBS Lett 388:139–142

Brummell DA, Balint-Kurti PJ, Harpster MH, Palys JM, Oeller PW, Gutterson N (2003) Inverted repeat of a heterologous 3′-untranslated region for high-efficiency, high-throughput gene silencing. Plant J 33:793–800

Burgess DG, Ralston EJ, Hanson WG, Heckert M, Ho M, Jenq T, Palys JM, Tang KL, Gutterson N (2002) A novel, two-component system for cell lethality and its use in engineering nuclear male-sterility in plants. Plant J 31:113–125

Cazzonelli CI, Velten J (2008) In vivo characterization of plant promoter element interaction using synthetic promoters. Transgenic Res 17:437–457

Cazzonelli CI, McCallum EJ, Lee R, Botella JR (2005) Characterization of a strong constitutive mung bean (*Vigna radiata L.*) promoter with a complex mode of regulation in planta. Transgenic Res 14:941–967

Chakravorty D, Botella JR (2007) Over-expression of a truncated *Arabidopsis thaliana* heterotrimeric G protein γ subunit results in a phenotype similar to α and β subunit knockouts. Gene 393:163–170

Danthinne X, van Emmelo J (1990) Studies on the translational properties of STNV RNA non-coding regions. 42nd International symposium on crop protection. Gent, Belgium

De Amicis F, Patti T, Marchetti S (2007) Improvement of the pBI121 plant expression vector by leader replacement with a sequence combining a poly(CAA) and a CT motif. Transgenic Res 16:731–738

De Loose M, Danthinne X, Van Bockstaele E, Van Montagu M, Depicker A (1995) Different 5′ leader sequences modulate β-glucuronidase accumulation levels in transgenic *Nicotiana tabacum* plants. Euphytica 85:209–216

Dunsmuir P (1985) The petunia chlorophyll a/b binding-protein genes - a comparison of *Cab* genes from different gene families. Nucleic Acids Res 13:2503–2518

Fornara F, Parenicova L, Falasca G, Pelucchi N, Masiero S, Ciannamea S, Lopez-Dee Z, Altamura MM, Colombo L, Kater MM (2004) Functional characterization of OsMADS18, a member of the AP1/SQUA subfamily of MADS box genes. Plant Physiol 135:2207–2219

Futterer J, Hohn T (1996) Translation in plants - rules and exceptions. Plant Mol Biol 32:159–189

Gallie DR (1993) Posttranscriptional regulation of gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol 44:77–105

Gallie DR, Walbot V (1992) Identification of the motifs within the tobacco mosaic virus 5′-leader responsible for enhancing translation. Nucleic Acids Res 20:4631–4638

Harpster MH, Townsend JA, Jones JDG, Bedbrook J, Dunsmuir P (1988) Relative strengths of the 35S cauliflower mosaic virus, 1′, 2′, and nopaline synthase promoters in transformed tobacco sugarbeet and oilseed rape callus tissue. Mol Gen Genet 212:182–190

Joshi JP, Zhou H, Huang X, Chiang VL (1997) Context sequences of translation initiation codon in plants. Plant Mol Biol 35:993–1001

Kawaguchi R, Bailey-Serres J (2005) mRNA sequence features that contribute to translational regulation in Arabidopsis. Nucleic Acids Res 33:955–965

Klaff P, Riesner D, Steger G (1996) RNA structure and the regulation of gene expression. Plant Mol Biol 32:89–106

Kozak M (1989) The scanning model for translation: An update. J Cell Biol 108:229–241

Laurena AC, Magdalita PM, Hidalgo MSP, Villegas VN, Mendoza EMT, Botella JR (2002) Cloning and molecular characterization of ripening-related ACC synthase from papaya fruit (*Carica papaya L.*). In: Proceedings of the International Symposium on Tropical and Subtropical Fruits

Lodish HF (1976) Translational control of protein synthesis. Annu Rev Biochem 45:39–72

Matsui T, Hori M, Shizawa N, Nakayama I, Shinmyo A, Yoshida K (2006) High-efficiency secretory production of peroxidase C1a using vesicular transport engineering in transgenic tobacco. J Biosci Bioeng 102:102–109

Mylne J, Botella JR (1998) Binary vectors for sense and antisense expression of Arabidopsis ESTs. Plant Mol Biol Report 16:257–262

Narsai R, Howell KA, Millar AH, O'Toole N, Small I, Whelan J (2007) Genome-wide analysis of mRNA decay rates and their determinants in *Arabidopsis thaliana*. Plant Cell 19: 3418–3436

O'Keefe DP, Tepperman JM, Dean C, Leto KJ, Erbes DL, Odell JT (1994) Plant expression of a bacterial cytochrome P450 that catalyzes activation of a sulfonylurea pro-herbicide. Plant Physiol 105:473–482

Odell JT, Nagy F, Chua N (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a ß-glucoronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol 31:805–813

Purnell MP, Botella JR (2007) Tobacco isoenzyme 1 of NAD(H)-dependent glutamate dehydrogenase catabolizes glutamate in vivo. Plant Physiol 143:530–539

Trusov Y, Zhang W, Assmann SM, Botella JR (2008) Heterotrimeric G protein Gγ-deficient mutants do not recapitulate all phenotypes of Gβ-deficient mutants. Plant Physiol 147:636–649

Trusov Y, Sewelam N, Rookes JE, Kunkel M, Nowak E, Schenk PM, Botella JR (2009) Heterotrimeric G proteins-mediated resistance to necrotrophic pathogens includes mechanisms independent of salicylic acid-, jasmonic acid/ethylene- and abscisic acid-mediated defense signaling. Plant J 58:69–81

Turner RL, Glynn M, Taylor SC, Cheung MK, Spurr C, Twell D, Foster GD (1999) Analysis of a translational enhancer present within the 5′-terminal sequence of the genomic RNA of potato virus S. Arch Virol 144:1451–1461

Venter M (2007) Synthetic promoters: genetic control through *cis* engineering. Trends Plant Sci 12:118–124

Wells DR, Tanguay RL, Le H, Gallie DR (1998) HSP101 functions as a specific translational regulatory protein whose activity is regulated by nutrient status. Gene Dev 12:3236–3251

Wu KQ, Hu M, Martin T, Wang CM, Li XQ, Tian LN, Brown D, Miki B (2003) The cryptic enhancer elements of the tCUP promoter. Plant Mol Biol 51:351–362

Zaccomer B, Haenni AL, Macaya G (1995) The remarkable variety of plant RNA virus genomes. J Gen Virol 76: 231–247