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Comparison of different constitutive and inducible promoters for the overexpression of transgenes in *Arabidopsis thaliana*

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Abstract

We compared the organ specificity and the strength of different constitutive (CaMV-35S, CaMV-35Somega, *Arabidopsis* ubiquitin *UBQ1*, and barley leaf thionin *BTH6* promoter) and one inducible promoter (soybean heat-shock promoter *Gmhsp17.3*) in stably transformed *Arabidopsis thaliana* plants. For this purpose we constructed a set of plant expression vectors equipped with the different promoters. Using the *uidA* reporter gene we could show that the CaMV-35S promoter has the highest expression level which was enhanced two- to threefold by the addition of a translational enhancer (TMV omega element) without altering the organ specificity of the promoter. The barley leaf thionin promoter was almost inactive in the majority of lines whereas the ubiquitin promoter exhibited an intermediate strength. The heat-shock promoter was inducible up to 18-fold but absolute levels were lower than in the case of the ubiquitin promoter. Conclusive quantitative results for different organs and developmental stages were obtained by the analysis of 24 stably transformed lines per promoter construct.

Introduction

For the overexpression of foreign proteins in *Arabidopsis thaliana* plants we were looking for a strong promoter. The CaMV-35S promoter has been shown to be active in most tissues of several plant species [e.g. 2, 3]. Although a CaMV-35S promoter with omega element is often used for such studies, the strength and the organ specificity of this promoter have not been analysed in stably transformed *A. thaliana* plants. The TMV (tobacco mosaic virus) omega element has been shown to be an efficient translational enhancer for different systems [9] but the level of enhancement differed between species and between transient and stable transformations [12]. Since it was neither known if this translational enhancer gives indeed a substantially higher expression level if used with the CaMV-35S promoter in stably transformed *A. thaliana* plants, nor if this element might alter the organ specificity of the CaMV-35S promoter, we decided to compare the strength and the organ specificity of the CaMV-35S promoter with that of the CaMV-35S omega promoter. We included in this comparison some other potentially useful promoters: the *UBQ1* ubiquitin promoter from *A. thaliana* [6], the inducible heat-shock promoter *Gmhsp17.3* from soybean [22] and the *BTH6* promoter from a barley leaf thionin gene (Holtorf *et al.*, unpub-

lished results). We constructed a set of expression vectors which differ only in the promoter. They have a *Nco* I site (with ATG codon for translational fusions) and five 3′-restriction sites for the insertion of coding sequences to be expressed. The *uidA* gene [18] was used as a reporter gene. To our knowledge, such a study has not been done before and yields conclusive results as to the organ specificity and strength of the different promoters in stably transformed *A. thaliana* plants.

Materials and methods

Construction of expression vectors

All vectors are based initially on pRT104 [24]. For promoters other than the CaMV-35S promoter the vector was cut with *Hin*d II and *Nco* I and the large vector fragment was isolated. The different promoters were then ligated to this fragment to yield promoter-terminator constructs.

The CaMV-35S omega promoter was amplified by PCR from the vector pJD330 (obtained from Dr. G. Galili, Rehovot, Israel) with the following primers: T7 (5′-AAT ACG ACT CAC TAT AG-3′) and OMA (5′-CGG AAT TCC ATG GTG TAA TTG TAA ATA GTA A-3′). The PCR fragment was cloned into pUC18/*Sma* I to give pSH8. This vector was digested with *Bam* HI (filled in with Klenow) and *Nco* I and the promoter fragment was ligated into the vector backbone from pRT104 to yield pSH9.

The *A. thaliana* ubiquitin promoter from *UBQ1* [6] was obtained as a 2500 bp fragment from Dr. R. Vierstra (Madison, WI). A *Nco* I site was introduced by PCR. Primers UBQ.1 (5′-CGG AAT TCA CGC GTA CAT TGA CAT ATA-3′) and UBQ.2 (5′-CGG AAT TCA GAT CTC CAT GGT TTG TGT TTC-3′) were used to amplify a 80 bp fragment from the downstream border of the ubiquitin promoter. This fragment was exchanged for the 80 bp *Mlu* I-*Bgl* II fragment from *UBQ1*, thereby introducing a *Nco* I site at the translation start site. The modified promoter was cut out with *Hin*d III (filled in with Klenow) and *Nco* I and ligated into the vector backbone from pRT104 to yield pSH5.

The promoter from the soybean heat-shock gene *Gmhsp17.3* [22] was obtained from Dr F. Schöffl (Tübingen, Germany). A 350 bp fragment containing all the necessary elements for heat-shock induction [22] was amplified with the primers HSP.1 (5′-CAC AAG ACT GAT AAG AGA CCA TGG AG-3′) to introduce an *Nco* I site and M13 -47 SP (5′-CAG CAC TGA CCC TTT TGG GAC CGC-3′). The PCR fragment was cloned into pUC18/*Sma* I and sequenced. Finally, the promoter was excised with *Bam* HI (filled in with Klenow) and *Nco* I and inserted into the pRT104 backbone to give pSH7.

A 1.5 kb promoter fragment from the barley leaf thionin gene BTH6 (Holtorf *et al.*, unpublished results) was cut out with *Bam* HI (filled in with Klenow) and *Nco* I and cloned into the pRT104 vector backbone to yield pSH6.

The *uidA*-coding sequence from pRAJ275 (Clontech) which has a *Nco* I site surrounding the start codon was provided with a *Bam* HI site at the 3′ end. The vector was first cut with *Eco* RI, filled in with Klenow, and *Bam* HI linkers were added. After this an intron was introduced [26]. The intron was amplified with primers INT.1 (5′-CGG AAT TCT ACG TAA GTT TCT GCT TCT ACC-3′) and INT.2 (5′-CGG AAT TCA GCT GCA CAT CAA CAA ATT TTG G-3′) from the vector p35SGUSINT [26] and first cloned into pUC18/*Sma* I (pSH2). From pSH2 the intron was excised with *Sna* BI and *Pvu* II and cloned into the *Sna* BI site of the *uidA*-gene. One clone with the correct sequence and orientation of the intron (pSH4) was further used to isolate the *Nco* I-*Bam* HI *uidA* intron fragment, which was cloned into the corresponding *Nco* I and *Bam* HI sites of the different promoter-terminator constructs to yield the complete GUS expression cassettes. These were excised with *Hin*d III and inserted into the *Hin*d III site of pBIN19 [4].

Transformation of Agrobacterium tumefaciens

A. tumefaciens strain LBA4404 containing the helper plasmid pLBA4404 (Clontech) was transformed with the pBIN19 constructs according to the method of Holsters *et al.* [15].

Transformation of A. thaliana

A. thaliana ecotype C24 was transformed by the root transformation method [25] essentially as described by Huang and Ma [16]. Excised shoots were transfered to test tubes to raise seeds. 40 to 50 individual transformants were generated for each construct. Integration of the *uidA*-cassettes was verified by GUS staining and PCR. 24 transgenic lines were chosen randomly for each construct and kanamycin-resistant T1 plants were raised to produce T2 seeds for further experiments.

Plant growth conditions

Plants were grown on MS medium [20] including 1% sucrose with 0.8 % agar or in soil in a growth chamber under long-day conditions (16 h light, 8 h darkness) at 20 °C or 22 °C. For GUS assays and *in situ* stainings plants were grown on MS agar.

Heat-shock conditions

Plants were transfered to liquid MS medium and heat-shocked for 2–5 h at 35 °C unless otherwise indicated. Plants in soil were heat-shocked in a growth chamber equipped with a fan by raising the temperature from 20 °C to 38 °C for four hours.

GUS assays

For each line 40 T2 plants (12-day old seedlings) or the leaves/roots of 3 T2 plants (4-week old plants with rosettes) were pooled. GUS activity was determined according to Jefferson *et al.* [18] and expressed as pmols or nmols MU per minute per mg protein. Protein concentration was measured as described by Bradford [5].

In situ GUS staining was done according to Jefferson *et al.* [18] with 5-bromo-4-chloro-3-indolylglucuronide (X-gluc). Plants were destained in 70% ethanol.

Results

Cloning of expression vectors

We wanted to compare different promoters for their suitability to express foreign genes in *Arabidopsis thaliana*. For this purpose it was necessary to have a set of expression vectors that differ only in the promoter. They were constructed as described in Materials and methods. *Nco* I (providing an ATG start codon) and *Bam* HI sites are used to clone the coding sequences which are to be expressed into these vectors (other possible 3′ restriction sites instead of *Bam* HI are: *Sst* I, *Kpn* I, *Eco* RI, *Xba* I). The different promoters/vectors were tested with an intron containing *uidA* gene [26]. The final constructs used in this study are shown in Fig. 1. They were transformed into *A. thaliana* as described in Materials and methods.

Optimizing heat-shock conditions

We first determined the optimal temperature for heat-shock induction of 4-week old leaves in liquid MS medium. As shown in Fig. 2, the optimal temperature under these conditions (2 h heat-shock) is 35 °C. This can also be seen in Fig. 3 (L), where the leaves of four different lines were stained for GUS activity after heat-shock for 2 h. Only at the optimal temperature a strong and regular staining can be seen, whereas a few degrees below this temperature the staining is only patchy. At 38 °C the four lines react differently, two showing a regular and strong staining, and the other two showing only a staining in the middle of the leaves around the midvein. The same optimal temperature was found for roots (Fig. 2) and seedlings (data not shown). Thus, there is only a rather narrow temperature window if one wants to achieve an optimal induction of the soybean heat-shock promoter. If the heat-shock was administered in air (for soil-grown plants) instead of liquid medium, the treatment had to be maintained for a longer period (at least 4 h) and a higher temperature (38 °C) was needed to achieve a comparable GUS level (data not shown).

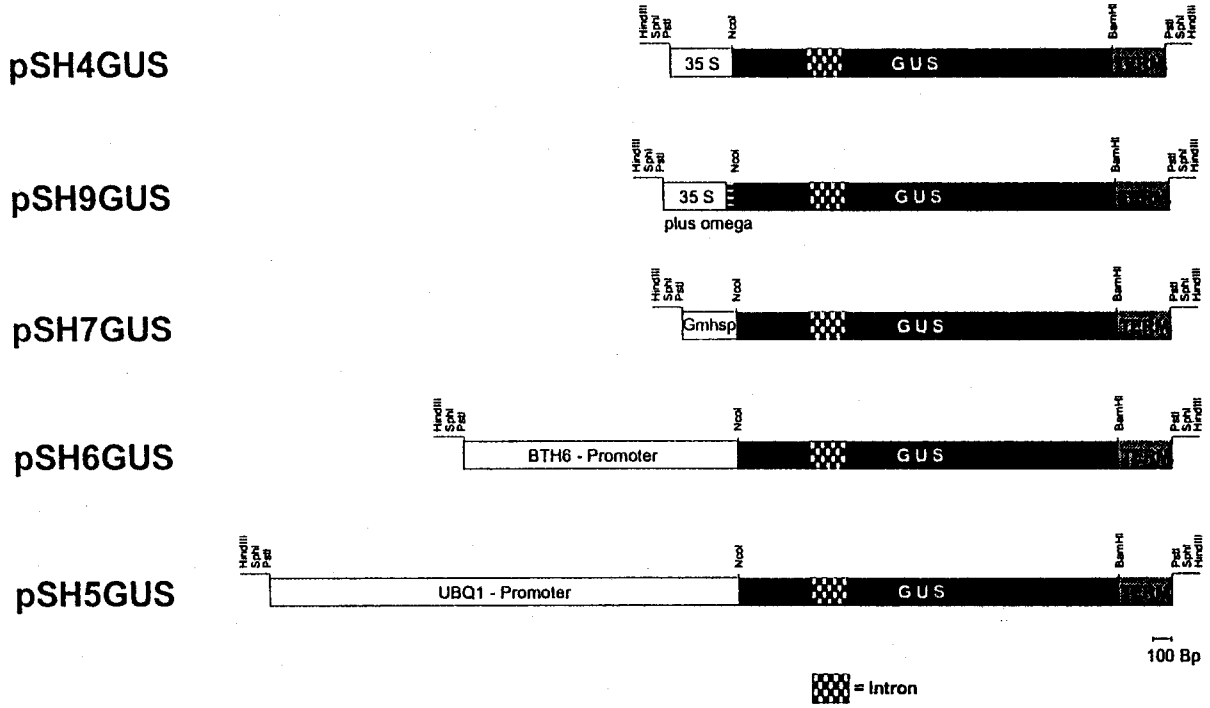

Fig. 1. Promoter-*uidA* constructs. Promoters are shown in white, the *uidA* gene (GUS) in black (intron depicted as checkered box), the CaMV terminator (TERM) in grey, and the omega element is striped.

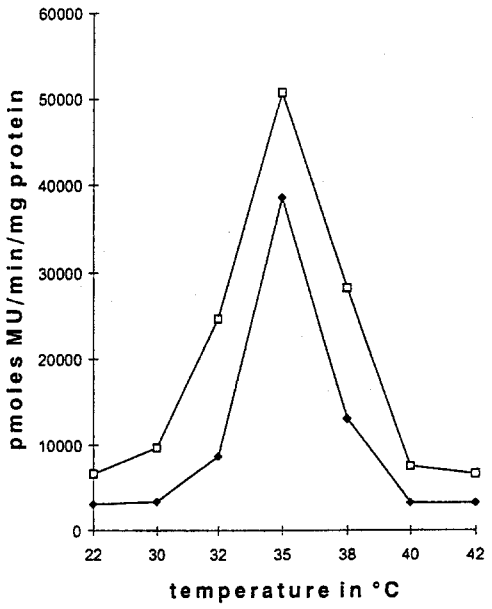

Fig. 2. GUS expression levels after heat-shock (2 h in liquid medium) at different temperatures in leaves (black rhombes) and roots (white squares). Values shown are the means of 4 independent experiments.

Organ specificity of the promoters

The overall appearance of X-gluc-stained *A. thaliana* plants expressing the 35S-*uidA* construct, the 35Somega-*uidA* construct, the *UBQ1-uidA* construct, and the induced heat-shock-*uidA* construct is about the same. Strong GUS activity is always found in the roots, cotyledons, leaves, and all parts of the inflorescence (Fig. 3). Only slight differences can be seen between the four promoters macroscopically. The staining of plants carrying the *UBQ1-uidA* construct is very regular as opposed to the patchy appearance of the 35S/35Somega-*uidA* plants. In addition, *UBQ1-uidA* seedlings show a stronger staining of the hypocotyl (Fig. 3, C). The *BTH6* promoter is characterised by a different expression pattern. Leaves of strong expressing lines (see below) show a high GUS expression but no GUS activity can be detected in the roots and in the hypocotyl (Fig. 3, D). In the inflorescences only the pollen grains are stained (Fig. 3, Q).

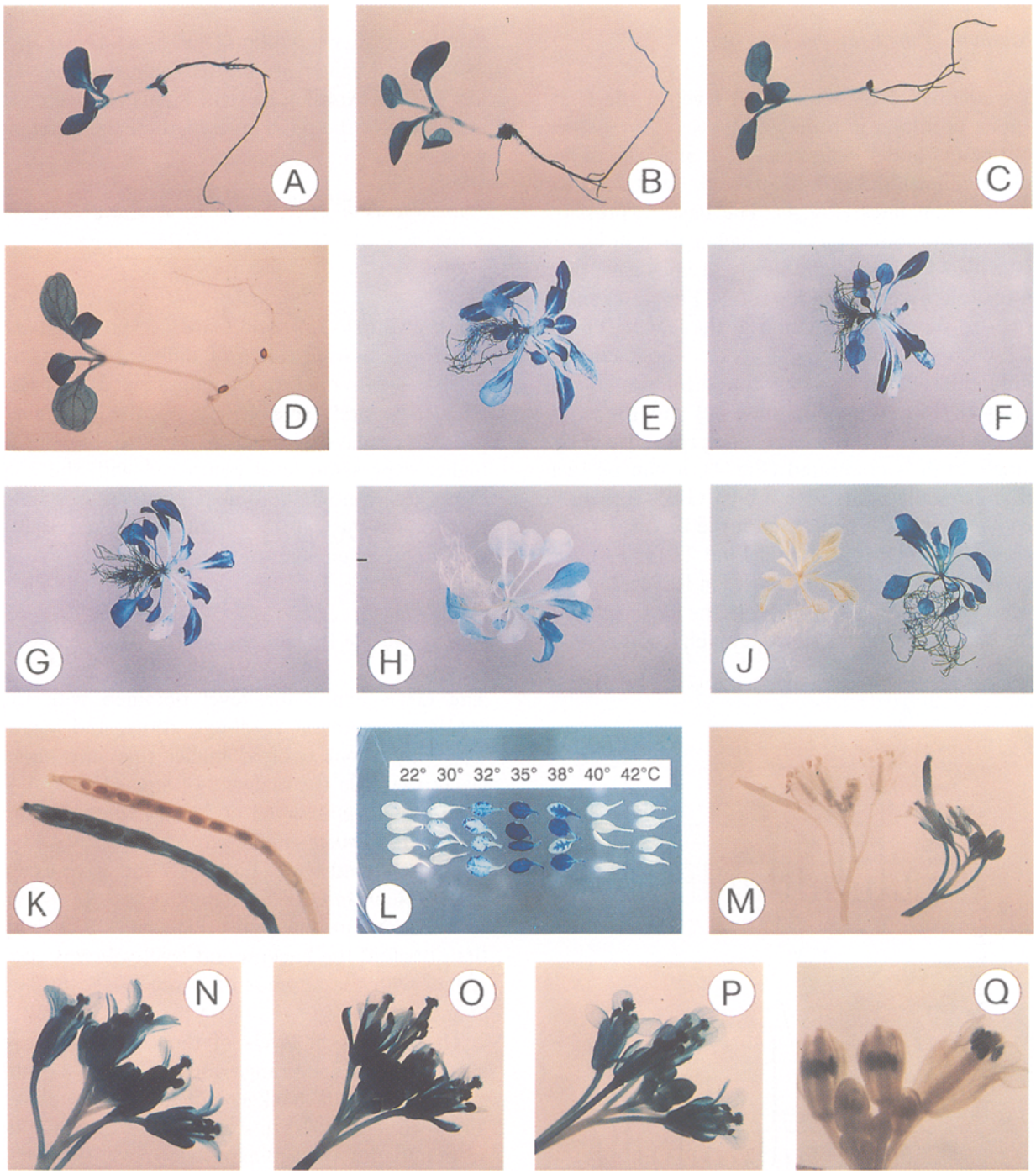

Fig. 3. In situ staining of transgenic *A. thaliana* plants with X-gluc. A – D, seedlings; E – J, 4-week old plants; K, pods; M – Q, inflorescences. Plants carry the CaMV-35S promoter (A, E, N), the CaMV-35Somega promoter (B, F, O), the *UBQ1* promoter (C, G, P), the BTH6 promoter (D, H, Q), and the heat-shock promoter (J – M). J, K, M show induced and non-induced plants or plant parts. In Q the signal in the anthers is only due to staining of the pollen grains. L shows the heat-shock response of leaves from 4 week old plants from 4 different lines after 2 h heat-shock at the respective temperature.

Strength of the heat-shock promoter

We compared the expression level of the heat-shock promoter in roots and leaves from 4-week old plants under heat-shock (5 h at 35 °C in liquid medium) and non-heat-shock conditions for 24 different lines (Fig. 4). The basal expression level in the roots is usually higher than in leaves (see also Fig. 2). The majority of lines gave only a twofold induction in the roots (Fig. 4, A) and a two- to threefold induction in the leaves (Fig. 4, B) when subjected to heat-shock. The maximum induction, which was only found for some lines, is about 15-fold in roots (line 22) and 18-fold in leaves (line 11). If the heat-shock response of the single lines is compared (Fig. 4), it can be seen that generally a line with a high GUS activity in roots, also has a high level in the leaves, but the correlation is not absolute. Line 23 for instance gives the highest GUS activity in leaves, but not in roots. The highest activity in the roots is found for line 22. Seedlings showed much lower expression levels after induction (Fig. 5, A). (Note that in Fig. 5 all values are plotted such that the values decline from left to right.) This gives a clear indication of the relative strength of the different promoters.

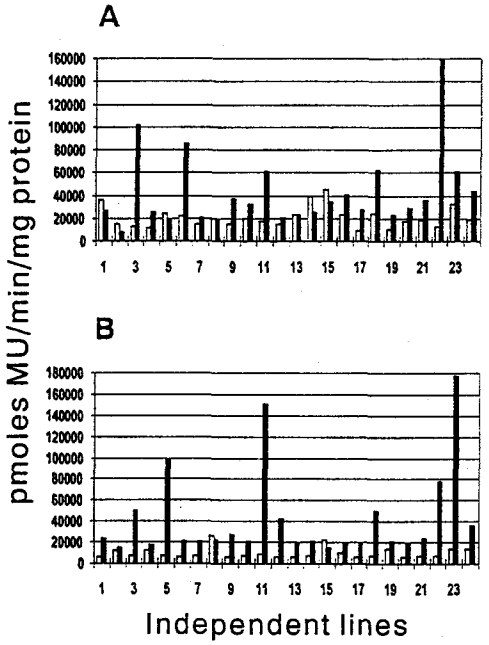

Fig. 4. Heat-shock induction (5 h 35 °C) in roots (A) and leaves (B) of the heat-shock promoter-*uidA* construct in 24 individual transgenic *A. thaliana* lines. Values for non-induced (white bars) and induced (black bars) plants are shown. (Note that values are in pmol MU per minute per mg protein).

Strength of CaMV-35S/CaMV-35Somega promoters

For the CaMV-35S- and CaMV-35Somega-*uidA* constructs a wide range of different expression levels was observed. In seedlings (Fig. 5, A), roots (Fig. 5, B), and leaves (Fig. 5, C) the 35Somega promoter on average leads to a two to three times higher expression level compared with the 35S promoter without the omega element. For both promoters, the expression in leaves is roughly threefold higher than in roots (Fig. 5, D).

Strength of the BTH6 *and the* UBQ1 *promoter*

The GUS expression level obtained with the *BTH6* promoter in seedlings, roots, and leaves was only about twofold higher than the background level in the majority of lines (Fig. 5, A – C). Only three lines out of 24 had an expression level in the leaves which was significantly above the background level (Fig. 5, C) and gave easily detectable *in situ* GUS staining (Fig. 3, D + H). In the roots, no line had a higher GUS activity than twofold the background level which is consistent with the absence of *in situ* GUS staining (Fig. 3, D, H).

The expression levels of the *UBQ1*-lines are spread over a wide range and are mostly significantly above the background level in seedlings and 4 week old roots and leaves (Fig. 5, A–C). In seedlings, the average expression level is significantly lower than in older leaves or roots.

Comparison of all promoters

In Fig. 5 (A – C) the values for all promoters in seedlings (A), roots of 4-week old plants (B), and

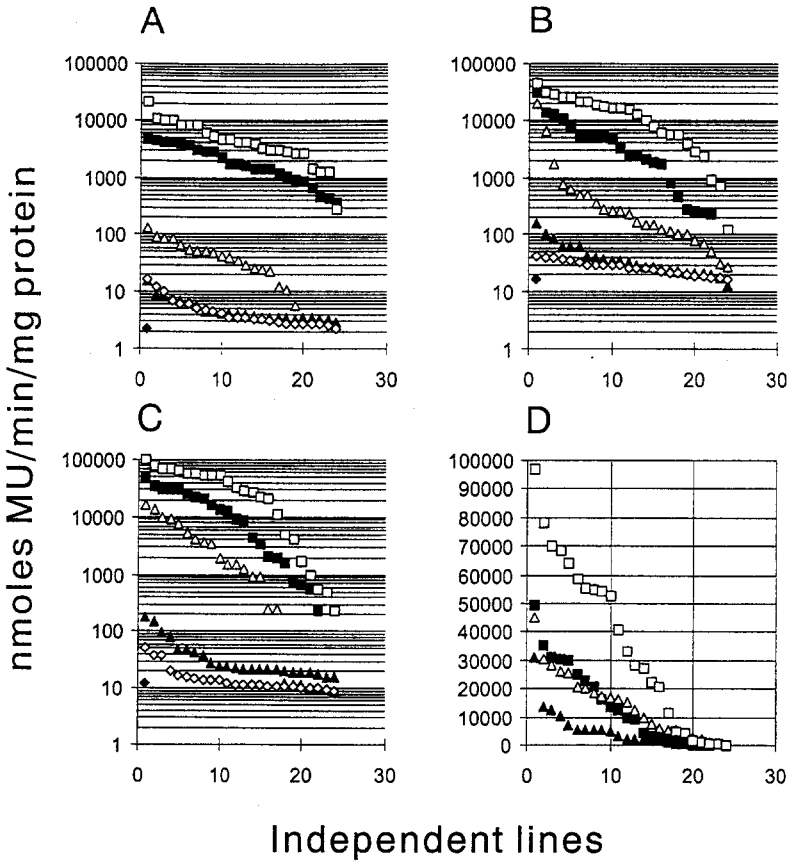

Fig. 5. GUS expression levels for 12-day old seedlings (A), as well as roots (B) and leaves (C) of 4-week old plants. 24 (22 in the case of the CaMV-35S promoter) independent transgenic *A. thaliana* lines carrying the CaMV-35Somega promoter (white squares), the CaMV-35S promoter (black squares), the *UBQ1* promoter (white triangles), the heat-shock promoter (induced, black triangle), and the *BTH6* promoter (white rhombes), respectively. The background value for wild-type plants is given as a black rhombus. D. Root (triangles) versus leaf (squares) GUS expression levels for the CaMV-35Somega promoter (white) and the CaMV-35S promoter (black) in 4-week old transgenic *A. thaliana* plants. (Note that all values are in nmol MU per minute per mg protein.

rosette leaves of 4-week old plants (C) are plotted together such that the GUS expression values decline from left to right. The *BTH6* promoter is the weakest promoter, followed by the induced heat-shock promoter (one heat-shock) and the *UBQ1* promoter. The strongest promoter is certainly the CaMV-35Somega promoter followed by the CaMV-35S promoter. The highest variation is found for the *UBQ1* promoter, especially in roots, where one line has a very high GUS expression level which is only exceeded by one 35S promoter line and some of the 35Somega lines (Fig. 5, B).

Discussion

Organ specificity of the promoters

In this study we have compared the strength and the organ specificity of five different promoters in *Arabidopsis thaliana*. As a reporter gene we used the *uidA* gene, which is well established for use in plants [18], and compared the organ specificity of the promoters by *in situ* staining with X-gluc (Fig. 3). No differences in organ specificity were found between the CaMV-35S and the CaMV-35Somega promoter. Both gave strong staining in

all plant parts but only a slight staining in the hypocotyl. In this regard the *UBQ1* and the induced heat-shock promoter were almost identical with the 35S promoters. The *BTH6* promoter was clearly different in its organ specificity, showing neither GUS staining in the roots and the hypocotyl, nor in the inflorescence except the pollen grains. About one out of 10 *BTH6* transgenic lines showed an expression pattern as shown in Fig. 3 (D, H, Q), whereas 9 out of 10 had a very low expression which was only visible in the petiole (data not shown). For the *in situ* staining, a strongly expressing *BTH6* line was used. The organ-specific expression of the *BTH6* promoter in *A. thaliana* is different from that observed in tobacco (Holtorf *et al.*, unpublished). In tobacco, the *BTH6* promoter is active in the whole inflorescence and, compared to *A. thaliana*, is stronger in the leaves and the stem.

Strength of the promoters

The root transformation method we used results in predominantly single-copy integrations [13] and only a limited number of tetraploid plants [1]. Thus, by using 24 independently transformed lines, the observed variation in the GUS expression level with the same construct is mainly due to the well known position effect which influences the expression of foreign genes in transgenic plants. Translational effects due to the constructs used (except the intended effect of the omega-element fused to the 35S promoter) and effects of the 3′ prime non-coding region [17] can be excluded since we used always the same *uidA*-intron construct, fused with the promoter at the *Nco* I site, and the same terminator.

The strength of the different promoters was compared in young seedlings and in roots and rosette leaves of 4-week old plants from 24 independently transformed lines. The highest levels were obtained for the CaMV-35Somega promoter followed by the CaMV 35S promoter in all organs that we tested (Fig. 5, A–C). The addition of a TMV omega-element (CaMV-35Somega) gave a two- to threefold enhancement of the expression level. In seedlings the best expressing 35Somega line had a sixfold higher expression compared to the best 35S line. It has been demonstrated that the omega element, the 5′-untranslated leader of TMV, functions as a translational enhancer *in vitro* and *in vivo* [10, 11, 12]. For tobacco and carrot protoplasts 30-fold enhancements of GUS activity have been reported [11]. Whether these very high values, five to ten times higher than in our system, are due to the different plant species used or due to the transient nature of the expression system used in that study, is not known. Nevertheless, the omega element seems to be a reliable translational enhancer also for *A. thaliana* and can give two- to threefold higher expression levels in transgenic plants without altering the organ specificity of the 35S promoter. Several other translational enhancers from other viruses are known [7, 9] and might have equal or even better effects in *A. thaliana* than the omega-element.

The other three promoters which we have used are all significantly weaker than the 35S promoter. Unexpectedly, the *BTH6* promoter from barley gave the lowest GUS expression levels. For this promoter only 3 out of the 24 lines analysed had a GUS expression level that was reliably detectable by *in situ* staining, whereas the GUS activity of the other lines remained at approximately the background level of wild-type plants. The reason for this is currently unknown. Although the *BTH6* promoter is very weak, a potentially desirable feature might be that it has a very low activity in roots which was undetectable by *in situ* staining in contrast to the four other promoters which have been tested. The *UBQ1* promoter from *A. thaliana* [6] proved to be of medium strength in our system. However, one line had a very high expression level in the roots; higher than the majority of the 35S lines (Fig. 5, B). Another noticeable feature of this promoter is that it gave a very regular GUS staining with X-gluc compared to the 35S promoters which often appeared very patchy (data not shown). The *UBQ1* promoter has also been tested in tobacco [6] and there its expression was found to be only slightly lower than that of the 35S promoter.

The heat-shock promoter from soybean [22] was included as an inducible promoter and could indeed be regulated by heat-shock in our system, but only about 2 out of 10 lines showed a good heat-shock response, the maximum induction being about 18-fold. The uninduced expression level is higher in roots as compared to leaves (Fig. 4). Only a very low heat-shock induction was found in seedlings and the promoter had a very low induced expression level comparable to that of the *BTH6* promoter. As has been previously observed with an *A. thaliana* heat-shock promoter [23], the optimal temperature for heat-shock in *A. thaliana* was 35 °C in liquid medium. The optimal temperature was higher with air incubation of plants grown in soil (data not shown). A disadvantage of the heat-shock promoter is that heat-shock leads to drastic alterations in the physiology of the whole plant [27]. Other potentially useful inducible promoters which might be used in *A. thaliana* have been described [8, 14, 19, 21] but no comparative study has been done yet.

Conclusions

We have constructed a set of plant expression vectors with an ATG start codon that differ only in the promoter. The omega element gives an two- to threefold enhancement of the CaMV-35S promoter in stably transformed *A. thaliana* plants and the CaMV-35Somega promoter is a very strong promoter for constitutive expression in all organs of *A. thaliana*.

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