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Expression of *gus* and *gfp* Genes in Garlic (*Allium sativum* L.) Cells Following Particle Bombardment Transformation

Cristiano Lacorte[1,2*], Daniella Barros[1]

[1]*EMBRAPA-Hortaliças, Br 060, Km 09, C.P. 218, CEP 70359 970, Brasília, DF, Brazil;* [2]*Present address: EMBRAPA-Recursos Genéticos e Biotecnologia, Parque Estação Biológica, C.P. 02372, CEP 70770-900, Brasília, DF, Brazil.*

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Abstract

The activity of promoter sequences was evaluated in garlic cells using the β-glucuronidase (GUS) gene as a reporter. Histochemical GUS assay indicated transient GUS activity in leaf, callus and root cells 48 hours after particle bombardment transformation. Quantitative fluorometric assays in extracts of transformed leaves demonstrated that the CsVMV promoter induced the highest level of gene expression, which was, on average, ten fold the level induced by CaMV35S and by the *Arabidopsis Act2* promoters and two fold the level expression observed with a construct containing a double CaMV35S plus the untranslated leader sequence from AMV. No activity or very low levels were observed when cells were transformed with plasmids containing the typical monocot promoters, *Act1*, from rice or the *Ubi-1*, from maize. The green fluorescent protein (GFP) was also tested as a marker gene for garlic transformation. Intense fluorescence was observed in leaf, callus and root cells transformed with a construct containing the *gfp* gene under control of the CaMV35 promoter. No fluorescence was detected when the *gfp* was under control of the *Ubi-1* promoter.

Introduction

Garlic (*Allium sativum* L.) is an important vegetable crop grown in many countries, used as condiment and for its therapeutic and medicinal properties (Sendl, 1995). Garlic plants are usually agamic and are propagated vegetatively either by cloves or by inflorescence bulbils. Except for a few varieties, plants do not set flower or the flowers are sterile (Pooler and Simon, 1994). The variability is restricted to the available clones and genetic improvement by sexual crosses is usually not possible. Therefore, approaches such as somaclonal variation and genetic transformation are important alternatives for improving the variability in the available clones.

Plant genetic transformation evolves a number of cellular and molecular events such as DNA transfer, gene expression, selection of transformed cells and plant regeneration. Transient gene expression has been widely used to optimize DNA delivery parameters and to compare promoter sequences in fusion with reporter genes. The expression of reporter genes and the activity of promoters have been studied in some species of the order Liliales, including tulip, lily, onion and garlic (Wilmink et al., 1995; Eady et al., 1996; Myers and Simon, 1996; Barandiaran et al., 1998). Difficulties in achieving *Allium* transformation have been reviewed by Eady (1995). Among these are the large size of the *Allium* genome and the low regeneration rates from transformation competent cells. In addition, Barandiaran et al. (1998) did not detect transient gene expression in garlic cells and showed that the activity of endogenous nucleases was as a limiting factor transformation of garlic.

In this study, the activity of promoters derived from monocots, virus and dicots were compared in different garlic tissues, using the β-glucuronidase (GUS) gene as a reporter. The green fluorescent pro-

* Corresponding author, E-mail: clacorte@cenargen.embrapa.br
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tein (GFP) was also tested as a marker gene for garlic transformation.

Materials and Methods

Plant material and culture conditions

Garlic bulbs (cv. Caçador) were stored at 4℃ for approximately 3 months to break dormancy. The cloves were separated from the bulbs and the protective and storage leaves removed. The stems were trimmed at the basal part to remove necrotic areas and were disinfested with 1% (v/v) sodium hypochlorite, pH 7.0, for 15 min. After rinsing with sterile water for 5-6 times, the stems were cultivated on medium containing half the concentration of MS salts and organic compounds (Murashige and Skoog, 1962), supplemented with 3% sucrose and 0.6% agar. Media were adjusted to pH 5.8 before the addition of agar and autoclaved at 120℃ for 15 min. Cultures were incubated at 25±2℃, 16 h photoperiod, and 40-45 $\mu Em^{-2}s^{-1}$ of light intensity.

After 7 days, young leaves, roots, and the basal parts of sprouting leaves were excised and placed on the center of a 9 mm Petri dish with MS medium supplemented with 0.9 μM 2,4-Dichlorophenoxy acetic acid (2,4-D), 3% sucrose and 0.7% agar.

Leaf, root and callus explants were used as target for particle bombardment. Callus culture was obtained from leaf explants cultured in MS medium supplemented with 4.5 μM 2,4-D, 1.0 μM 6-(γ-γ-dimethylallylamino) purine (2-ip), 3% sucrose and 0.7% agar. The cultures were maintained in the dark and subcultured monthly. The effect of osmotic treatment on transient expression in callus was evaluated. Calli were transferred to media containing manitol (0, 0.1 or 0.5 M) one day before transformation and maintained on these media for another 2 days, before assaying for GUS activity.

Particle bombardment conditions

DNA was precipitated onto tungsten particles using the calcium/spermidine method (Klein et al., 1995; Aragão et al., 1996). Plasmid DNA (total of 5 μg) was added to 50 μL of tungsten (Sylvania, M10) particle suspension (60 mg/mL in 50% glycerol). DNA and particles were precipitated by adding 20 μL of 0.1 M spermidine and 50 μL of 2.5 M $CaCl_2$. The preparations were washed two times with absolute ethanol (150 μL) and resuspended in a final volume of 24 μL. To avoid large particle clumps the preparations were briefly sonicated (approximately 2 seconds) and distributed in 3 μL aliquots over the macrocarrier discs.

The DNA-coated particles were accelerated using a high-pressure helium device (Sanford et al., 1991), under partial vacuum of 25 inches Hg. The helium pressure used was 900 PSI, and the distance from the macrocarrier support to the target was approximately 10 cm. After transformation the explants were incubated at 25±2℃, under diffused fluorescent light (10-15 $\mu Em^{-2}s^{-1}$) with 16 h photoperiod.

Plasmid constructs

Plasmid DNA was isolated using the Qiagen Maxiprep kit. Relevant features of the constructs utilised are listed in Table 1. In experiments to compare the activity of different promoters, DNA preparations were adjusted to contain equimolar concentrations of the gus gene. According to the size of each plasmid,

Table 1. Gene constructs utilised and their relevant features.

Plasmid	Promoter	Coding region	Termiantor	Size[b](Kb)	References
pBI221	CaMV 35S	*gus*	*Nos*	5.7	Jefferson et al., 1987
pBI426	CsMV35S-35S-AMVE[a]	*gus:nptII*	*Nos*	6.2	Datla et al., 1991
pILTAB308	CsVMV	*gus*	*Nos*	5.3	Verdaguer et al., 1996
pAH27	*Ubi-1*	*gus*	*Nos*	7.0	Christiansen and Quail, 1996
pAct1-D	*Act1*	*gus*	*Nos*	8.0	McElroy et al., 1990
pBSGUS	*Act2*	*gus*	*Nos*	6.5	-[c]
PSGFP	CaMV 35S	*sgfp*	*Nos*	4.5	Chiu et al., 1996
PMNG1001	*Ubi-1*	*sgfp*	*Nos*	6.4	Upadhyaya et al., 1998

[a] AMVE = Alfalfa mosaic virus untranslated leader sequence
[b] Approximate size
[c] F. Arãgo, personal comunication

appropriate amounts of plasmid pBSSK- (Stratagene) was added to the preparation in order to obtain the same final mass of DNA per preparation (5 μg).

Rice explants

Rice explants were used as a control for transformation conditions. Rice seeds (cv. Tain) were disinfested in 1% sodium hipochloride for 10 minutes, washed thoroughly and sown on MS medium supplemented with 2.0 mg/L 2,4-D, 3% sucrose, and 0. 7% agar, pH 5.8. Seeds were maintained in the dark, at 25℃. After two days the embryos were removed and prepared for particle bombardment transformation as described for garlic explants.

Histochemical and fluorometric GUS assays

Transient GUS activity in leaf, callus and root explants was assayed two days after particle bombardment. For the histochemical assays, explants were incubated in 100 mM sodium phosphate buffer (pH 7.0), 0.1 % Triton, 10 mM Na_2EDTA, and 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), for 6-8 hours, at 37℃. After staining explants were rinsed in distilled water and fixed in 70% ethanol.

Quantitative fluorometric GUS assays were done essentially as described by Jefferson et al. (1987). Leaf explants were macerated in 400 μL of extraction buffer (100 mM sodium phosphate buffer (pH 7.0), Triton® 0.1%, 10 mM Na_2EDTA, 1 mM β-mercaptoethanol). The extract (20 μL) was mixed with 180μL of reaction buffer (1 mM 4-methyl-umbelliferone-β-D-glucuronide (MUG) in extraction buffer). Samples of 20 μL were taken at 15 min. intervals and transferred to 1.980 mL of stop buffer (0.2 M $NaCO_3$). Fluorescence was measured in a fluorometer (DyNAQuant™, BioRad) with an excitation/emission of 360/460 nm, using 4-Methyl umbellifone (4-MU) as a standard. Each experiment consisted of at least five repetitions. Data were statistically analyzed following the Student t-test. GUS specific activity expressed in nmol 4-MU/min/mg protein was determined after measuring protein concentrations using the BioRad kit based on the method of Bradford (1976).

GFP detection

GFP fluorescence was monitored under a Zeiss Axiovert 100 TV inverted microscope with UV light and a 450/520 excitation/emission filter set. Leaf explants were mounted on glass slides with water. Calli and roots were observed directly on the culture plates.

Results

Promoter activity

The activity of promoter-gene fusion constructs in extracts of garlic leaves was evaluated using the β-Glucuronidase (GUS) fluorometric assay. The promoter from the cassava vein mosaic virus (CsVMV) and the double 35S promoter from the cauliflower mosaic virus (CaMV-CaMV-AMV), in plasmid pBI 426, drove the highest levels of *gus* expression (Figure 1). GUS activity was barely detectable under the control of the actin (*Act1*) or ubiqutin (*Ubi-1*) promoters isolated from rice and maize, respectively. Intermediate levels of activity was observed when the explants were transformed with the CaMV 35S (plasmid pBI221) or the *Act2* promoter from *Arabidopsis* (plasmid pBSGUS) (Table 1, Figure 1).

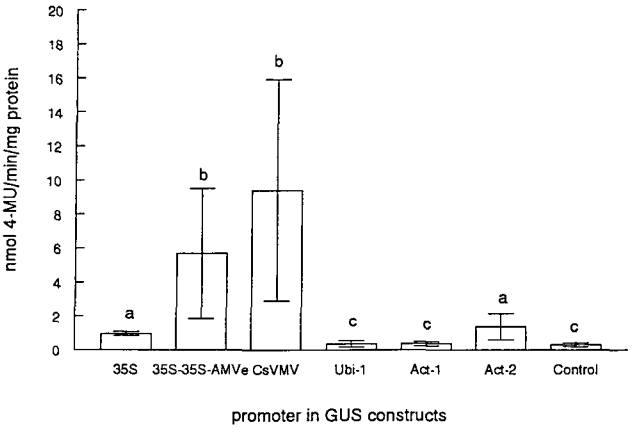

Figure 1. β-Glucuronidase activity in extracts of leaf explants two days after particle bombardment transformation with different promoter-gus constructs. Bars represent the standard deviation of the mean values. Means with the same letter are non-significantly different at P = 0.05.

Histochemical GUS assays in leaf explants transformed with plasmid pILTAB 308 confirm the high activity of the CsVMV promoter in this cells. A large number of intensely stained blue cells could be observed after two hours in reaction buffer (Figures 2A, 2C). When driven by *Ubi-1* or by *Act1* promoters, GUS activity was not significantly different from the non-transformed controls, under the conditions used in the fluorometric assays. However, in histochemical assays, we could observe 1-2 faint blue cells per explant transformed with the pAHA 27 constructs, containing the *Ubi-1* promoter (Figure 2D). To assure that plasmids and transformation were working properly, rice escutelum explants were

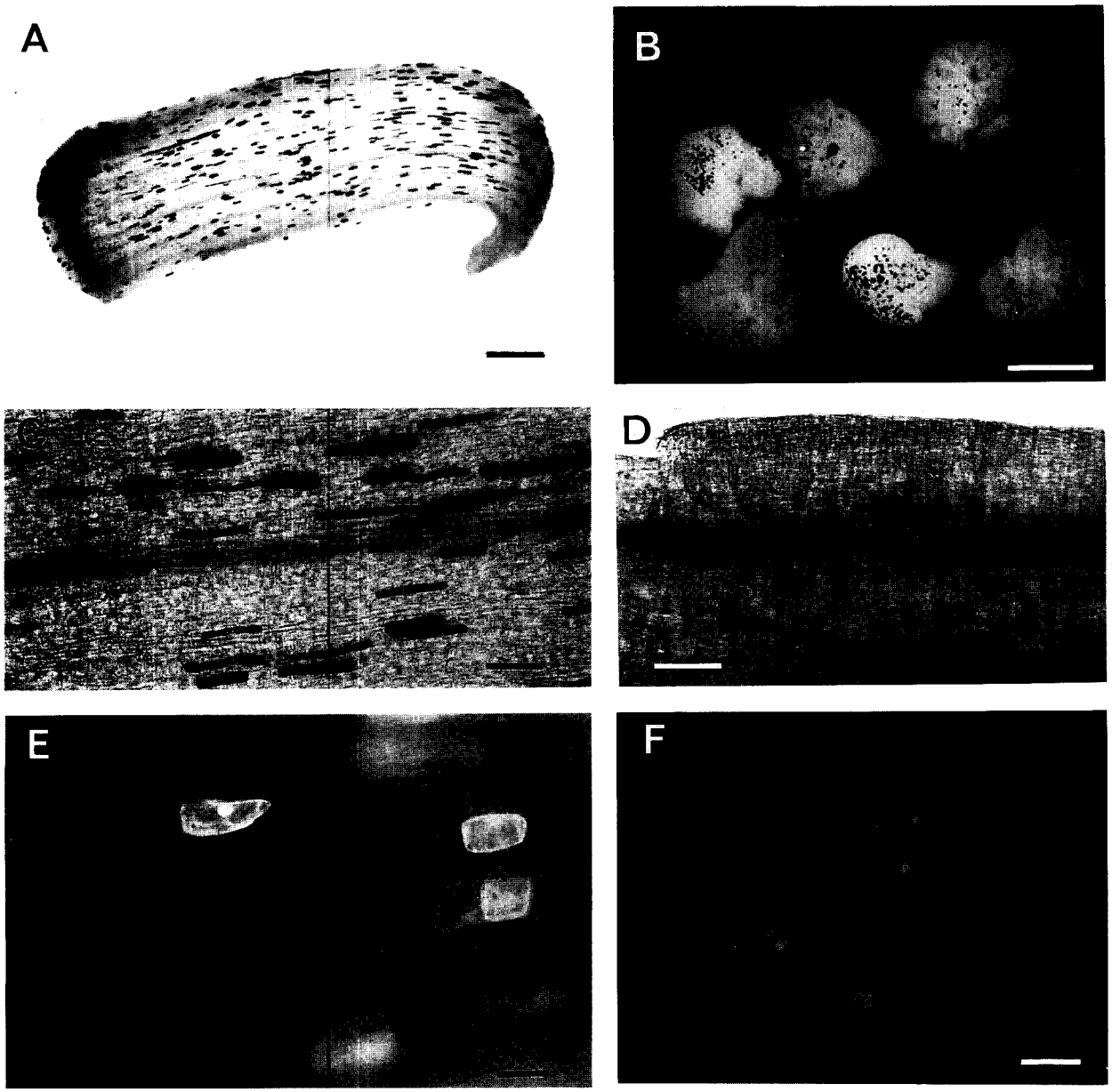

Figure 2. (A - D) Histochemical GUS assay in garlic leaf explants (A) and callus (B) two days after particle bombardment with plasmid pILTAB 308. (C) Leaf explant at higher magnification. (D) Leaf explant transformed with pAH27. (E and F) GFP fluorescence in leaf cells (E) and callus (F), two days after transformation with plasmid pSGFP. Bars = 1 mm in A and B and 100 m in C, D, E and F.

transformed together with garlic explants and analyzed by the histochemical GUS assay. Explants transformed with plasmids pAHA27 and pAct1 showed intensely stained blue cells and the number of transformed cells was higher than in explants transformed with the plasmid pBI221, containing the CaMV35S promoter (data not shown).

GUS activity in callus and root explants

Plasmid pILTAB 308 was used to transform callus an roots. Histochemical assays showed that the CsVMV promoter is also active in these cells (Figure 2B). The effect of a osmoticum treatment on callus transformation was also evaluated. The addition manitol to the culture medium did not increase the number of cells expressing GUS in relation to the calli cultured without the osmoticum treatment (data not shown).

Some endogenous GUS activity was observed in callus tissues. This activity was characterized by a diffused faint blue color and was easily distinguished from the exogenous β-glucuronidase activity of transformed cells. Moreover, this activity was negligible when potassium ferricyanide and ferrocyanide in concentrations over 2.5 mM were added to the reaction buffer. Thus, we routinely adopted a concentration of 5 mM which also reduced the diffusion of the reaction products in both callus and leaf explants (Figure 2B, 2C). GUS activity in garlic extracts was not inhibited by the addition of potassium ferricyanide and potassium ferrocyanide at concentrations up to 10 mM, as determined by fluorometric assays (data not shown).

GFP fluorescence

GFP fluorescence was detected in garlic leaf and callus cells transformed with the CaMV35S-*gfp* construct (Figures 2C, 2D). The GFP version used (SGFP) has a single excitation peak at 490 nm and emission at 510 nm and a green fluorescence 120-fold greater than the wild-type GFP (Chiu et al., 1996). A strong fluorescence was observed in transformed cells and practically no endogenous fluorescence in leaf tissues was detected (Figure 2C). Although some explants showed endogenous red fluorescence from chlorophyll, this apparently did not interfere with GFP fluorescence.

In callus tissues a pale-green background was present but fluorescence derived from the GFP-expressing cells was quite strong and clearly distinguishable (Figure 2D). Fluorescence could be observed as early as 4-5 hours after particle bombardment transformation and a few fluorescing cells could be identified for a period of up to four weeks.

No fluorescence was observed in garlic cells transformed with an *Ubi-1-gfp*, in either callus, root or leaf explants. In rice cells, strong fluorescence could be observed following transient expression of *gfp*, under the control of either CaMV35S or *Ubi-1* regulatory sequences (data not shown).

Discussion

We have demonstrated the transient expression of *gus* and *gfp* genes in garlic. GUS activity and GFP fluorecence were detected in leaf, callus and root cells. Quantitative β-glucuronidase assay was used to compare the activity of promoter sequences in leaf explants.

Transient expression of GUS in garlic cells was described recently by Barandiaran et al. (1998). These authors reported that GUS activity could only be detected after the tissues were treated with nuclease inhibitors. They found high level of endogenous nuclease activity in garlic extracts and suggested that the lack of transient *gus* expression was due to plasmid degradation (Barandiaran et al., 1998). Our results, in contrast, showed transient expression of both *gus* and *gfp* genes, whose products could be detected at high levels in different tissues such as leaf, callus and roots, without any treatment with nucleases inhibitors. Myers and Simon (1996) reported the expression of *gus* in cell cultures of garlic, and the addition of nuclease inhibitors was also not needed. Therefore, nuclease activity does not seem to be a general limiting factor for transient gene expression in garlic and the differences observed are likely to result from the varieties of garlic used.

Transient gene expression analysis has been widely applied to evaluate promoter activity and to establish and optimize transformation conditions. Although the expression and stability of a foreign gene in a transgenic plant probably evolve several factors such as position effect, gene silencing and other complex and yet unstudied events, transient expression analysis represents a valuable approach (Warkentin et al., 1992; Rathus et al., 1993; Schledzewski and Mendel, 1994; McElroy and Brettell, 1994; Upadhyaya et al., 1997). High and constitutive activity of promoter controlling the appropriate selectable gene is an important factor to obtain transgenic plants (McElroy and Brettell, 1994; Li et al., 1997).

Some of the promoters that are reported to drive constitutive expression in monocots and dicots where chosen and tested in garlic cells. The constructs tested contained virus-derived promoters, such as the CaMV35S and the CsVMV, the maize ubiquitin (*Ubi-1*), and the actin promoters, *Act1* and *Act2*, from rice and *Arabidopsis*, respectively (McElroy et al., 1990; Datla et al., 1991; Verdaguer et al., 1996; Christiansen et al., 1996; An et al., 1996).

Among these promoters, the CaMV35S is the most common and widely used for driving high and constitutive expression in transgenic dicot plants (Rathus et al., 1993; Schledzewski and Mendel, 1994; Wilmink et al., 1995). The activity of the CaMV35S promoter is further enhanced if two copies are placed in tandem (Datla et al., 1991), such as in plasmid pBI426. This plasmid also contains an untranslated leader sequence from the AMV (alfalfa mosaic virus), that was shown to increase gene expression approximately 20-fold in tobacco protoplasts (Datla et al., 1993). The CaMV35S is also functional and widely used in monocot species. However, the rice *Act1* and the maize *Ubi-1* promoter sequences have been reported to induce 10-50 fold the expression level relative to the CaMV35S in transient assays and in

transgenic plants of a number of monocot species, such as rice, maize, barley, sugar-cane, wheat, oats and fescue (reviewed by McElroy and Brettell, 1994 and by Wilmink et al., 1995)

The other virus-derived sequence we tested was the CsVMV promoter. This promoter was isolated recently from cassava vein mosaic virus (CsVMV) and characterized (Verdaguer et al., 1996). It presents a constitutive expression pattern in cassava and rice plants and its activity in tobacco plants and protoplasts is comparable to the CaMV35S (Verdaguer et al., 1996). In garlic cells, the CsVMV was the most active among the promoters tested. GUS expression under control of CsVMV promoter was approximately ten fold the observed with the CaMV 35S and two fold the double CaMV35S promoter. We also showed that this construct is active in leaf, callus and root cells and can also be considered as constitutive promoter in garlic.

The promoter sequences *Act1* and the *Ubi-1*, derived from rice or maize, respectively, were not active or induced scant levels of expression in garlic cells. However, when the *Act2* promoter sequence from *Arabidopsis* was tested (plasmid pBSGUS) the GUS activity was comparable to the CaMV35S promoter (Figure 1). These results suggests that dicot promoters are more active in garlic cells than promoter derived from monocots.

Wilmink et al. (1995) compared the activities of the *Act1, Ubi-1 Emu* and CaMV35S promoters in leek (*Allium ampeloprasum*), lily (*Lilium longiflorum*), tulip (*Tulipa gesneriana*), rice and *Nicotiana plumbaginifolia*. The activities of the *Ubi-1, Act1* and *Emu* promoters was much higher in rice cells than in leek, lily, tulip and *N. plumbaginifolia*. With the CaMV35S promoter, in contrary, the activity in rice cell was very low. Therefore, they concluded that the promoter activity in lily, leek and tulip resembles the pattern observed in *Nicotiana* (Wilmink et al., 1995). Our results and other reports with species of the Liliales also support this conclusion. In onion (*Allium cepa*), GUS activity was not detected under the control of the rice *Act1* promoter and activity under the CaMV35S was 5-10 fold the activity with the *Ubi-1* promoter (Eady et al., 1996). In garlic, results reported by Barandiaran et al. (1998), after treating the tissues with nuclease inhibitors, and by Myers and Simon (1996) in cell suspension also found that the expression under the control of the rice *Act-1* promoter was very low or absent and much higher under the control of the CaMV35S promoter. A direct comparison between these studies and our results could not be made because these authors did not quantitatively analyze GUS activity.

The *Act1* and *Ubi-1* constructs contain an intron located at the 5' untranslated region. The presence of these introns enhances gene expression in rice and maize cells but had an inhibitory effect in *Nicotiana* and leek, and sugarcane (Rathus et al., 1993; Eady et al., 1996; Christiansen and Quail, 1996). The low activity of the *Act1* and *Ubi-1* promoters in dicots and in some monocots from the Liliales was suggested to result from misleading of splicing or other differences in promoter sequence and its recognition by transcription factors (Rathus et al., 1993; Wilmink et al., 1995). The species from the Liliales so far studied, including garlic, may differ from cereal monocots in relation to intron recognition and splicing and behave more like a dicot. Our results with the *Act2* promoter, from *Arabdopisis*, corroborates with this hypothesis. The *Act2* promoter also harbors an intron which is probably recognized and processed in garlic cell, in contrary to the *Act1* promoter from rice. Differences in the intron recognition sites have been characterized between mono and dicots. However, detailed molecular analysis would be necessary to confirm whether the low activity of monocot promoters among species from the Liliales results from the regulatory sequence and the interaction with transcriptional factors, from intron recognition and processing, or from both. Nevertheless, our results are in accordance with previous studies on the use of cereal promoters as for their restrictions as gene expression vectors in monocot species from the Liliales.

The green fluoresce protein (GFP) was also tested as a marker for garlic cell transformation. The expression of GFP have been described in transient assays in orange, *Arabidopsis*, alfalfa, maize, rice, soybean, spruce and pine. Transgenic plant of tobacco (reviewed by Leffel et al., 1997). Transgenic plants of tobacco, *Arabidopsis*, rice, sugar-cane and maize have also been reported to express GFP (Leffel et al., 1997; Vain et al., 1998). The GFP is a vital marker, does not require any substrate or cofactors, can be fused to proteins and is relatively easy to be detected. These features make the *gfp* an attractive reporter gene with a broad application in cell biology (Haseloff and Amos, 1995; Cubitt et al., 1995; Leffel et al., 1997). The GFP has some disadvantages is relation to the β-glucuronidase, once it is not suitable for quantitative assays and is not as sensitive. Besides, in *Arabidopsis* high levels of GFP expression seemed to interfered in plant regeneration (Haseloff and Amos, 1995; Chiu et al., 1996).

We have detected strong GFP fluoresce in explants transformed with plasmid pSGFP, containing the *sgfp* gene under control of the CaMV35S promoter (Table 1). The *sgfp* is a synthetic gene, from which the cryptic intron sites have been altered and the cromophrore mutated at the position 65 from ser-

ine to threonine. The SGFP shows a single excitation peak at 490 nm and a 120-fold brighter green fluoresce than the wild type GFP (Chiu et al., 1996). Under blue light the endogenous fluoresce from leaves, callus or root cells was much lesser intense than if under shorter wave-length (i.e. 405 nm). No fluorescence was observed when the explants were transformed with the *Ubi-1-sgfp* construct (plasmid pMNG1001) and is in accordance with the results obtained with the *Ubi-1-gus* fusion (Figure 1).

GFP fluorescence was detected 4-5 hours after transformation and was still present in a few cells after four weeks. This suggests integration of the foreign DNA or that the GFP is very stable in garlic cells. We did not observe cell division among these GFP expressing cells. The explants were not cultured under condition that are optimal for inducing callus formation and no selection agent nor selection gene was included. Besides, the transformed cells showing GFP fluorescence, could possibly be non competent for division. Further experiments pursuing transformed callus and transgenic plants are undergoing and would confirm the suitability and value of utilizing the GFP as a vital marker for garlic transformation.

The results obtained on promoter activity shall be useful for developing a garlic transformation protocol. Likewise, the GFP can be used as a vital marker gene to follow transformation events. These factors, together with an efficient selection for the transformed cells and for plant regeneration are important steps towards a garlic transformation protocol and to ensure high levels of gene expression in transgenic plants.

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References

An Y-Q, McDowell JM, Huang S, McKinney EC, Chambliss S, Meagher RB (1996) Strong constitutive expression of the *Arabidopsis ACT2/ACT8* actin subclass in vegetative tissues. Plant J 10: 107-121.

Aragão FJL, Barros LMG, Brasileiro ACM, Ribeiro SG, Smith FD, Sanford JC, Faria JC, Rech EL (1996) Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed via particle bombardment. Theor Appl Genet 93: 142-150.

Barandiaran X, Di Pietro A, Mart n J (1998) Biolistic transfer and expression of a *uid*A reporter gene in different tissues of *Allium sativum* L. Plant Cell Rep 17: 737-741.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72: 248-254.

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Cur Biol 3: 325-330.

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY (1995) Undertanding, improving and using green fluorescent proteins. Tibs 20: 229-233.

Datla RSS, Bekkaouni F, Hammerlindl JK, Pilate G, Dunstan DI, Crosby WL (1993) Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci 94: 139-149.

Datla RSS, Hammerlindl JK, Pelcher LE, Crosby WL, Selvaraj G (1991) A bifunctional fusion between β-glucuronidase and neomycin phosphotransferase: a broad-spectrum marker enzyme for plants. Plant Sci 101: 239-246.

Eady CC (1995) Towards the transformation of onions (*Allium cepa*). N Zel J Crop Hort Sci 23: 239-250.

Eady CC, Lister CE, Suo Y, Schaper D (1996) Transient expression of *uidA* consructs in *in vitro* onion (*Allium cepa* L.) cultures following particle bombardment and *Agrobacterium*-mediated DNA delivery. Plant Cell Rep 15: 958-962.

Haseloff J, Amos B (1995) GFP in plants. TIG 11: 328-329.

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907.

Klein TM, Gradziel T, Fromm ME, Sanford JC (1988) Factors influencing gene delivery into *Zea mays* cells by high-velocity microprojectiles. Biotechnol 6: 559-563.

Leffel SM, Mabon SA, Stewart CN (1997) Applications of green fluorescent protein in plants. BioTechniques 23: 912-918.

Li Z, Upadhyaya NM, Meena S, Gibbs AJ, Waterhouse PM (1997) Comparison of promoters and selectable marker genes for use in Indica rice transformation. Molecular Breeding 3: 1-14.

McElroy D, Brettell RS (1994) Foreign gene expression in transgenic cereals. TIBTech 12: 62-68.

McElroy D, Zhang W, Cao J, Wu R (1990) Isolation of an efficient actin promoter for use in rice transformation. Plant Cell 2: 163-171.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tisue cultures. Physiol Plant 15: 473-497.

Myers M, Simon P (1993) Callus induction in garlic and its implication for transformation. HortScience 28: 489.

Pooler MR, Simon PW (1994) True seed production in garlic. Sex Plant Reprod 7: 282-286.

Rathus C, Bower R, Birch R (1993) Effect of promoter, intron and enhancer elements on transient gene expression in sugar-cane carrot protoplasts. Plant Mol Biol 23: 613-618.

Sanford JC, Devit MJ, Russell JA, Smith FD, Harpending

PR, Roy MK, Johnston SA (1991) An improved, helium-driven biolistic device. Technique 3: 3-16.

Schledzewski K, Mendel RR (1994) Quantitative transient gene expression: comparison of promoters for maize poliubiquitin 1,rice actin 1,maize-derives Emu and CaMV 35S in cells of barley, maize and tobacco. Transgenic Research 3: 249-255.

Sendl A (1995) *Allium sativum* and *Allium ursinum*: Part 1 - Chemistry, analysis, history, botany. Phytomedicine 4: 323-339.

Upadhyaya, NM, Ramm K, Gaudron J, Craig S, Wang MB, Gupta S, Okita TW, Waterhouse PM (1998) Can GFP replace *uidA* as a reporter gene to monitor transformation of cereals by biolistic or *Agrobacterium*? In: PJ Larkin (Ed.), Proceedings of the 4th AsiaPacific Conference on Agricultural Biotechnology, pp. 111-113.

Vain P, Worland B, Kohli A, Snape JW, Christou P (1998) The green fluorescence protein (GFP) as a vital screenable marker in rice transformation. Theor Appl Genet 96: 164-169.

Verdaguer B, de Kochko A, Beachy RN, Fauquet C (1996) Isolation and expression in transgenic tobacco and rice plants, of the cassava vein mosaic virus (CVMV) promoter. Plant Mol Biol 31: 1129-1139.

Warkentin TD, Jordan MC, Hobbs SLA (1992) Effect of promoter-leader sequence on transient reporter gene expression in particle bombarded pea (*Pisun sativum* L.) tissues. Plant Sci 87: 171-177.

Wilmink A, van de Ven BCE, Dons JJM (1995) Activity of constitutive promoters in various species from the Liliacea. Plant Mol Biol 28: 949-955.