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Short communication

5′-Regulatory region of *Agrobacterium tumefaciens* T-DNA gene 6b directs organ-specific, wound-inducible and auxin-inducible expression in transgenic tobacco

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Abstract

The regulatory activity of a 826 bp DNA fragment located upstream of the pTiBo542 TL-DNA gene 6b coding region was analysed in transgenic tobacco, using β-glucuronidase (*gus*) as a reporter gene. The region was shown to drive organ-specific, wound- and auxin-inducible expression of the reporter, the effect being dependent on the type and concentration of auxin.

The soil bacterium *Agrobacterium tumefaciens* causes crown gall tumours on many plant species. The biochemical and molecular genetic basis of this phenomenon was extensively studied (for review see [13]). After transfer of a specific DNA segment (T-DNA) of tumour-inducing (Ti) plasmid from the agrobacterial to the infected plant cell genes carried on the T-DNA are expressed that disrupt the cell metabolism. Three genes (*iaaH*, *iaaM* and *ipt*) provide for the biosynthesis of the phytohormones auxin and cytokinin, which leads to plant cell proliferation and formation of a tumor; gene 5 modulates the auxin response of tumor cells, and gene 6b remains the only one gene of the 'core' T-DNA for which the role in crown gall tumorigenesis is still poorly understood. It participates in the regulation of the tumor cell responses to auxins and cytokinins [25, 28–30]. We have previously cloned [24] the 5′-regulatory region (826 bp from the start of translation) of gene 6b from the TL-DNA of pTiBo542 [12], fused it to the *gus*-coding region and the 3′-regulatory region of the gene coding for nopaline synthase (*nos*) and transferred this fusion gene

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X89966.

into tobacco plants (*Nicotiana tabacum* L. cv. Petit Havana SR1) via the *Agrobacterium* binary vector system. We also produced transgenic tobacco plants carrying the *gus*-coding region under the control of the 35S-e promoter, the CaMV 35S promoter enhanced by the duplication of the enhancer region (EP 0 339009 A2) and the *nos* 3'-regulatory region. Analysis of GUS activity in roots and young leaves of the primary transformants revealed that the P6b-*gus* plants contain 20–80-fold higher GUS activity in roots than in young leaves [3]. Here we report a more detailed analysis of the activity of the 5'-regulatory region of gene 6b in different organs of the transgenic plants, as well as the effect of wounding and phytohormones on the activity of the regulatory region.

We analysed GUS activity in 40-day-old kanamycin-resistant seedlings of the F1 transgenic tobacco plants carrying the P6b-*gus* fusion (Table 1). The highest GUS activity was found in roots, followed by cotyledons and hypocotyls.

We also determined GUS activity in different organs of the P6b-*gus* and P35S-e-*gus* plants grown *in vitro* to the 12-leaf stage (Table 2, Fig. 1). The results indicated that the 6b 5'-regulatory region provided maximal GUS activity in roots, followed by stems and leaves. We found a gradual increase of GUS activity from the shoot apex towards the base in stems and leaves of the P6b-*gus* plants. The basipetal gradient of GUS activity is not simply the result of the accumulation of GUS, since P35S-e-*gus* plants did not show this pattern of expression (Fig. 1, Table 2). The expression profiles of P6b-*gus* and P35S-e-*gus* fusions differed considerably (Fig. 1). P6b-*gus* plants showed much lower GUS activities in roots, upper and middle leaves, but higher activi-

Table 1. GUS activity in seedlings of F1 transgenic plants carrying the P6b-*gus* fusion.

Number of the independent transgenic line	GUS activity, nmol MU per minute per mg protein		
	roots	hypocotyls	cotyledons
11	28.7	0.8	4.4
20	10.2	0.1	0.8
21	22.3	0.3	0.9
45	18.9	0.1	1.2
51	56.5	1.0	0.2
60	27.8	0.3	1.9

For each independent transgenic line roots, hypocotyls and cotyledons of 30 seedlings grown on MS medium [24] containing 100 mg/l of kanamycin were pooled and used for extract preparation. Explants were frozen in liquid nitrogen and stored at − 70° C until analysis. Plant extracts were prepared and GUS activity was measured fluorometrically according to Jefferson *et al.* [16]. Protein content of the extracts was determined by the method of Bradford [5]. BSA was taken as a protein standard. Experimental variations in the GUS activity values in all our assays reported here did not exceed 10% of an average value. MU-4-methylumbelliferone.

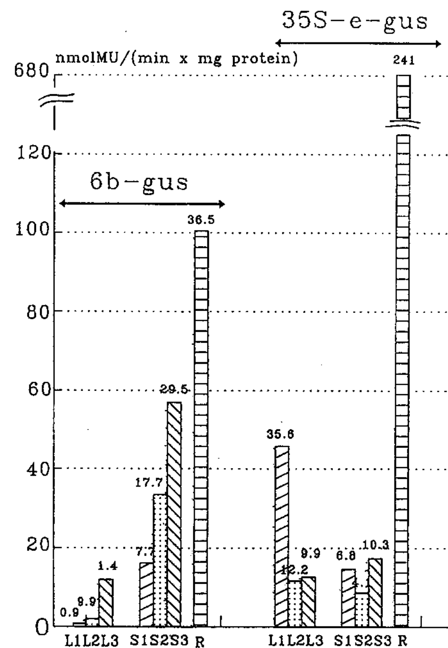

Fig. 1. The average values of the GUS activity in the different organs of F1 P6b-*gus* and P35S-e-*gus* plants. For each independent transgenic line one representative F1 plant grown on MS medium containing 100 mg/l of kanamycin was taken. Extracts were prepared from upper leaf (the 1st leaf from the apex, 2.5–3.5 cm long) (L1), middle leaf (the 5th or 6th leaf from the apex, 4.5–6.5 cm long) (L2), lower leaf (the 12th leaf from the apex, 3–4 cm long) (L3), stem sections (2–3 mm thick) (S1, S2, S3) and roots (R). Leaf explants were taken avoiding veins; stem sections were taken from the internodes just below the site of attachment of the respective leaf; roots were taken as total. This figure represents the average values for eight independent P6b-*gus* transformants and six independent P35S-e-*gus* transformants, standard deviations are shown as a numbers above the respective bars.

Table 2. GUS activities in the different organs of F1 P6b-*gus* and P35S-e-*gus* plants, nmol MU per minute per mg protein.

Plant No	Leaves			Stem			Root
	upper	middle	lower	upper	middle	lower	
P6b-*gus*							
10	0.5	2.4	26.0	9.1	40.8	67.4	75.0
21	0.6	0.7	1.3	15.9	26.7	56.3	66.7
38	0.7	4.2	26.7	22.2	62.5	120.0	120.0
45	0.3	0.6	12.7	13.3	25.0	47.6	132.0
11	3.0	3.0	4.4	27.3	38.0	66.6	93.3
20	0.2	1.3	1.0	6.2	10.5	22.2	68.6
51	0.6	3.6	18.5	26.7	53.8	57.0	175.8
60	0.1	0.4	5.5	9.1	10.2	18.6	72.7
P35S-e-*gus*							
24	108.1	37.6	27.8	19.4	12.5	29.6	686.0
32	16.9	2.4	1.9	6.6	3.3	7.4	762.0
81	42.3	8.3	11.6	16.3	11.1	20.0	593.0
82	31.7	8.2	12.4	18.4	8.6	17.4	1025.0
12	73.6	12.5	22.2	22.9	13.4	28.6	785.0
13	2.4	1.0	0.3	4.5	3.2	1.5	228.0

For each independent transgenic line one representative F1 plant grown on MS medium containing 100 mg/l of kanamycin was taken. The explants of different organs were taken as described in the legend to Fig. 1. GUS activity in the extracts prepared from the explants was determined as described in the legend to Table 1.

Table 3. Distribution of GUS activity within a mature leaf of P6b-*gus* plants, nmol MU per minute per mg protein.

Plant No	Leaf blade	Middle vein	Petiole
10	2.4	12.2	46.3
11	2.6	15.5	22.4
20	1.3	3.5	10.7
21	0.7	6.1	14.8
38	4.2	16.8	34.3
45	0.6	4.4	9.1
51	3.6	17.8	29.4
60	0.4	3.0	5.4

We analysed GUS activity in the extracts prepared from different parts of a mature leaf (5th or 6th leaf from the apex, 4.5–6.5 cm long). GUS activity was determined as described in the legend to Table 1.

Table 4. Distribution of GUS activity within a flower of P6b-*gus* plants, nmol MU per minute per mg protein.

Plant No	Corolla	Stamens	Ovary	Stigma and style	Sepal	Leaf
10	29.7	11.5	0.08	24.7	2.2	0.7
11	9.5	20.0	0.15	10.4	2.8	2.4
20	62.7	52.4	0.08	21.1	2.1	1.3
21	20.7	12.5	0.16	19.2	1.3	0.6
38	26.9	9.2	0.10	9.8	1.6	1.7
51	46.7	0.4	0.07	11.6	4.8	1.8
60	29.2	10.5	0.04	10.0	2.1	2.0

For each independent transgenic plant grown on soil substitution mixture we pooled different organs of three flowers the 1st-2nd day after anthesis. We also analysed GUS activity in the 1st leaf from the inflorescence. GUS activity was determined as described in the legend to Table 1.

ties in middle and lower stem than P35S-e-*gus* plants.

GUS activity was differentially distributed within a mature leaf. In the leaves of the P6b-*gus* plants the lowest GUS activity was found in the leaf blades, while middle veins showed a higher GUS activity, followed by petioles (Table 3). We also analysed GUS activity in flowers of the P6b-*gus* primary transformants (Table 4). GUS activity was differentially distributed within a flower with a maximum in the corolla and a minimum in the ovary.

It is worth to be noted that we also found GUS activity in the cells of the *Agrobacterium* strain carrying the binary vector plasmid containing the P6b-*gus-nos* 3′ fusion, the strain we used to produce the transgenic plants. GUS activity was not found in the cells of the original *Agrobacterium* strain B6S3-SE [7] which we used as a *vir* helper in plant transformation. Expression in *Agrobacterium* was earlier found for some other T-DNA genes [9, 10, 15].

It was shown for 6b genes from different Ti plasmids (pTiAch5, pTiC58 and pTiTm4) that they participate in the regulation of cellular responses to auxins and cytokinins [25, 28–30]. As we reported previously, the activity of the pTiBo542 6b 5′-regulatory region was stimulated by placing explants of mature transgenic leaves on callus-including medium containing 0.5 mg/l of naphthaleneacetic acid (NAA), and 0.2 mg/l of benzylaminopurine (BAP) [3]. In the present study we analysed the effect of different auxins and cytokinins in various concentrations on the

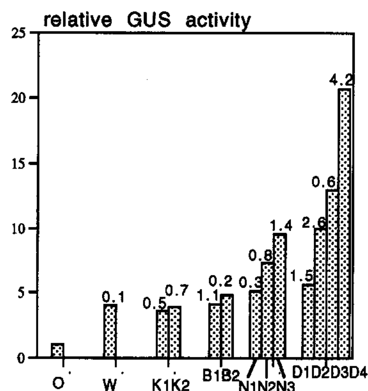

Fig. 2. Effect of auxin and cytokinin on the activity of the 6b 5′-regulatory region. Mature leaves of 12-leaf stage plants grown on soil substitution mixture were cut into ca. 0.5 cm^2 sections and the slices were floated on sterile water or solutions of auxins or cytokinins for 22 h. GUS activity was measured in extracts of the leaf fragments before (O) and after incubation (W, in water; K1, in solution containing 0.1 mg/l kinetin; K2, 5.0 mg/l kinetin; B1, 0.1 mg/l BAP; B2, 5.0 mg/l BAP; N1, 0.1 mg/l NAA; N2, 2.0 mg/l NAA; N3, 5.0 mg/l NAA; D1, 0.1 mg/l 2,4-D; D2, 0.5 mg/l 2,4-D; D3, 1.0 mg/l 2,4-D; D4, 5.0 mg/l 2,4-D). The ratio between the GUS activity in leaf fragments incubated in a given solution and the GUS activity in the leaf fragments before incubation was calculated for each independent transgenic plant. The figure represents the average values for six independent P6b-*gus* transformants; standard deviations are shown as a numbers above the respective bars.

activity of the 6b 5′-regulatory region (Fig. 2) (all phytohormone concentrations used in the analysis fall within a working range of concentrations used in plant tissue culture; catalogue 'Sigma. Plant Cell Culture', 1990). GUS activity in leaf explants of P6b-*gus* plants was enhanced by auxin treatment, with the effect depending upon the type and the concentration of auxin. 2,4-dichlorophenoxyacetic acid (2,4-D) had a more significant effect than NAA. The enhancement of GUS activity increased with the auxin concentration. No significant effects were observed with any of cytokinins tested, including BAP, kinetin (see Fig. 2), *trans*-zeatin and isopentenyladenine (data not shown). GUS activity in the leaf explants incubated in sterile water was also increased (Fig. 2 and 3a) suggesting wound inducibility of the 6b 5′-regulatory region activity. Wounding of leaves of the intact P6b-*gus* plants grown *in vitro* also induced 6b-driven GUS activity, although to a lesser extent (Fig. 3b). The 35S-e promoter activity was not influenced by phytohormones or wound treatment (data not shown).

Organ-specific activity in transgenic plants was observed earlier for the promoters of many other T-DNA genes, including *nos* [2], octopine synthase gene (*ocs*) [8, 19], mannopine synthase gene (*mas*) [21, 27], agropine synthase gene (*ags*) [14],

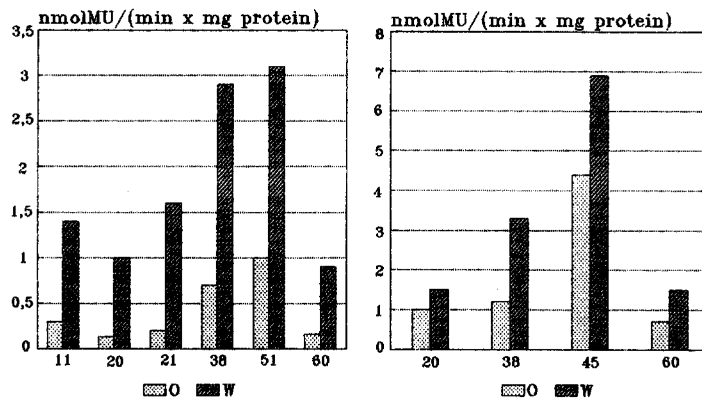

Fig. 3. Wound induction of the 6b 5′-regulatory region activity in leaves of P6b-*gus* plants. a (left). GUS activity in the extracts of the leaf explants before (O) and after (W) floatation on sterile water for 22 h (see legend to Fig. 2). b (right). GUS activity in the extracts of a leaf before wounding (O) and of the same leaf left on the plant for 22 h after wounding (W); F1 plants grown *in vitro* to 12-leaf stage were used in the analysis, wounding was carried out by making two holes (5 mm diametre) in the leaf blade (avoiding veins) of a mature leaf. After 22 h, 1 mm thick rings around the holes were taken and analysed for GUS activity.

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                      ocs-like element region                distance
                                                          ocs - TATA, bp
                        T              CGA  G      T AA
"ocs" consensus  TG ACG  AA          G       T ACG
                        C              GAC  T      C CC

gene 6b          AA ACGCACCATTTGCGCTGATTG AA                  218
    ocs          AA ACGTAA          GCGCTTACGT AC             141
    mas2'        TG ACGCTC          GCGGTGACGC CA              39
    nos          TG AGCTAA          GCACATACGT CA              85
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Fig. 4. Sequence homologous to the *ocs* 16 bp palindrome found in the 6b promoter region and the homologous sequences from *ocs*, *mas* 2′ and *nos* promoters. The consensus sequence for the *ocs* element, derived from the alignment of *ocs*-like elements of the promoters of seven opine synthase genes of T-DNAs from Ti and Ri plasmids and three plant viral genes, was taken from the paper by Bouchez *et al.* [4]. The 5 bp gap was introduced into the consensus sequence, *ocs*, *mas2′* and *nos* sequences to align them with the 6b sequence.

gene 5 [18], isopentenyltransferase gene (*ipt*) [6, 26]. Most of the studied T-DNA promoters are preferentially active in roots and lower parts of transgenic plants, which may be associated with the fact that these are the usual sites of agrobacterial infection in mature. The expression characteristics of the 6b 5′-regulatory region resemble those of the other auxin-inducible T-DNA promoters, especially those of *nos* and *mas*: the highest activity in the roots of transgenic plants, basipetal pattern of expression in the aerial parts of the plants, wound inducibility and auxin inducibility. However, the *mas* promoter was also inducible by cytokinin (BAP), albeit to a lesser extent, than by auxin (NAA) [1, 2, 21]. It would be interesting to elucidate *cis*-regulatory elements within the 6b 5′-flanking region responsible for its differential activity and compare them with known elements [4, 11, 19, 20, 23] from the promoters of other T-DNA genes. We have found in the 6b 5′-regulatory region a sequence with partial homology to the 16 bp palindromic element of the *ocs* promoter (Fig. 4); sequences homologous to the *ocs* element have been identified previously for 6 other T-DNA genes and some viral genes [4]. The importance of the sequence for the 6b promoter function remains to be determined.

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