Plant Molecular Biology **17**: 711–725, 1991.

Regulation of *Agrobacterium tumefaciens* T-*cyt* gene expression in leaves of transgenic potato (*Solanum tuberosum* L. cv. Désirée) is strongly influenced by plant culture conditions

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Received 28 August 1990; accepted in revised form 16 June 1991

Key words: Agrobacterium tumefaciens, crown gall, gene expression, genomic position effects, *Solanum tuberosum* L., T-*cyt*.

Abstract

The promoter region of the *Agrobacterium tumefaciens* T-*cyt* gene was linked in a translational fusion to the coding DNA of the reporter gene *uidA* (for β-glucuronidase or GUS protein; EC 3.2.1.31) and to *nos* 3′ flanking DNA. The chimaeric gene was introduced by *Agrobacterium* transformation into potato (*Solanum tuberosum* L. cv. Désirée). In nine transgenic lines, the average GUS levels were highest in extracts from stems and roots of *in vitro* grown plants (*ca.* 11000 GUS activity units per pmol MU per mg protein per min) but lower in leaves of the *in vitro* grown plants (*ca.* 7000 units). GUS activity was intermediate in stems and roots of plants grown in soil as well as in *in vitro* crown galls (*ca.* 3000 units). Activity was low in tubers, irrespective of whether these developed *in vitro* or in soil (both *ca.* 100 units), and lowest of all in leaves of soil-grown plants (*ca.* 10–15 units). However, in shoot cultures re-established from soil-grown plants, GUS activity in the leaves increased to that determined in the original shoot cultures. Hence, plant culture conditions strongly influenced the expression of the T-*cyt-uidA-nos* gene. In particular, it was silenced in leaves of soil-grown plants. The results are compared with previous analyses of the promoter region of the wild-type T-*cyt* gene and with the growth properties of a large number of crown gall cell lines and crown-gall-derived plants, including over forty *S. tuberosum* cv. Désirée cell lines isolated in the present study that were transformed with the wild-type T-*cyt* gene and six promoter-mutated derivatives. A number of implications are discussed for crown gall formation and for control of expression of plant genes which contain *Activator* or G-box type 5′ expression control sequences.

Introduction

The importance of environmental conditions for regulation of gene expression in plants is well established. Extensively studied examples include the light-regulated photosynthetic genes coding for the small subunit of rubisco (rbcS), the chlorophyll *a*/*b*-binding proteins of the light-harvesting complex, and phytochrome [12, 17, 37]. In general, the environmental stimuli are converted into controlled gene expression via transcription initiation complexes targeted at, and interacting with, arrays of short 5′-flanking sequences.

The movement of plants between *in vitro* and soil conditions represents an environmental switch which is important in plant conservation and micropropagation, but which is less well studied by molecular approaches. An extreme example of a reversible change in plant phenotype that occurs in such transfers, and which has a monogenic basis, is in plants transformed with *Agrobacterium tumefaciens* shoot-inducing T-DNA or in plants transformed with its T-*cyt* gene alone [for reviews see 5, 19, 28]. The T-*cyt* locus, also known as *ipt*, *tmr*, *roi* and gene *4*, codes for dimethyl allyl pyrophosphate 5′ AMP isopentenyl transferase [1, 3, 6] which catalyses the first committed step in cytokinin biosynthesis [24].

The changes induced by the T-*cyt* gene in transformed cells or plants vary, but a typical example is in potato where transformed cell lines maintained as shoot cultures *in vitro* have short, frequently branching shoots with callus at their base from which multiple shoots develop but, generally, no roots [32, 34]. The phenotype is therefore quite distinct from that of untransformed potato shoots. Grafting of the transformed shoots onto root stocks of normal potato and continued culture *in vitro* does not alter shoot phenotype, despite the development of a normal (untransformed) root system [33]. Transfer to soil, however, causes a gradual change in the phenotype of the scions, albeit in amenable transformed lines only. The plants become phenotypically more like untransformed potato plants [32, 36]. Re-establishment of the lines as *in vitro* cultures restores the contrast in *in vitro* phenotypes. Associated with the phenotypic normalization upon plant transfer to soil culture conditions are reductions in T-*cyt* steady-state RNA and cytokinin levels as well as various changes in the *in vitro* translation products of extracted mRNAs [7, 33]. Similar observations have been made in other plants, in particular in earlier isolated transformed tobacco lines [36, 38].

Deletion analysis of 5′ T-*cyt* flanking DNA using a tumour induction assay [13, 14, 15] showed that the T-*cyt* promoter spans only 184 bp and that the most important promoter sequences for normal T-*cyt* gene expression are a 13 bp '*Activator*' element at around −160 bp upstream of the ATG start codon and one of two TATA boxes at around positions −73/−67 and −42/−33. The *Activator* element has 77% homology with the core sequence of animal enhancers and substantial homology with 5′ flanking DNA regions in many plant genes, including short DNA regions with demonstrated importance, such as in soybean β-conglycinin gene expression [14, 15]. It is also homologous to the 8 bp dyad G-box found in 5′ flanking DNA of rbcS and alcohol dehydrogenase (Adh) genes which has been analyzed by gel retardation, by *in vivo* DMS footprinting and by chimaeric gene expression in transgenic plants [16, 17, 18]. A *trans*-acting factor(s) for the G-box is present in cell cultures and leaves of *Arabidopsis* and shows *in vivo* binding in the cell cultures, but not in the leaves [27].

Here we show that T-*cyt* 5′ flanking DNA, linked in a translation fusion to the reporter gene *uidA*, when introduced in transgenic potato gives high GUS activities in organs and tissues of *in vitro* grown plants, but is silenced to near background levels specifically in the leaves of soil-grown plants. To enable a better comparison between the results of the current T-*cyt-uidA-nos* chimaeric gene expression analysis and the distinct phenotypes of crown gall cell lines and crown-gall-derived plants, as caused by the introduction of T-*cyt* genes, we also introduced the wild-type T-*cyt* gene and six promoter-mutated derivatives into the same genetic background of potato cv. Désirée, and examined the phenotypes

of the regenerated cell lines and plants, when the plants were grown *in vitro* and in soil [34]. An additional reason for isolating these lines was to obtain plant materials that would be of potential use in examining how the introduced T-*cyt* genes gave alterations in plant growth and development as a way towards studying the natural cytokinin-related control mechanisms [34].

Materials and methods

*Plasmid construction and transformations using a chimaeric T-*cyt-uidA-nos *gene*

A chimaeric gene consisting of 5′ T-*cyt*-flanking DNA, some T-*cyt*-coding DNA, *uidA*-coding DNA and *nos*-3′ flanking DNA was constructed. Briefly, a 575 bp *Bam* HI fragment from pRAL3401 [13] was cloned into pUC18 and then subcloned, in the desired orientation as a *Hin*d III-*Sma* I fragment into a derivative of pUC18 already containing *uidA* DNA subcloned from pBI101.2 [21, 22]. Thus, the constructed plasmid, pTIP203, had a translational fusion between T-*cyt* and *uidA-nos* 3′ DNA which was verified by sequencing over the fusion site using an oligonucleotide primer specific to *uidA* 5′ coding DNA. From pTIP203 the chimaeric T-*cyt-uidA-nos* gene was subcloned as a 2.6 kb *Eco* RI/*Hin*d III fragment into pBIN19 [4] to give pTIP206. All molecular manipulations were according to Maniatis *et al.* [25]. Plasmid pTIP206 was transformed into *Escherichia coli* strain JM83 and conjugated by triparental mating, using *E. coli* strain HB101(pRK2013) into *A. tumefaciens* strain LBA4404(pAL4404), ultimately giving LBA4404 (pTIP206; pAL4404). This *Agrobacterium* strain was used to isolate kanamycin resistant transgenic derivatives of potato (*Solanum tuberosum* L. cv. Désirée) by *in vitro* stem segment transformation as described previously [40].

Putative transformed plants were confirmed for resistance to kanamycin by assessing root and shoot growth from 3–5 nodal stem segments on media with 50 and 200 mg/l kanamycin respectively. Lines with confirmed resistance were identified and plants multiplied by micropropagation on Murashige and Skoog (MS) medium (Flow Labs) supplemented with 20 g/l sucrose and solidified with 9% (w/v) agar (Difco-Bacto agar). The plants were maintained *in vitro* on supplemented solid MS medium without added kanamycin [33]. The regenerated plants were either morphologically identical to shoot cultures of Désirée or showed relatively minor differences (e.g. DT206B3 had slightly deformed leaves). Nine lines were randomly chosen, each from an independent transformation event, for analysis of GUS activity.

*Transformations using wild-type and mutant T-*cyt *genes*

A number of wild-type and promoter-mutated T-*cyt* genes, isolated previously [14, 15], were used to obtain correspondingly transformed potato plants. The genes were maintained in the wide-host-range plant transformation vector pAGS113 [41] in *Agrobacterium* host LBA4404 (pAL4404) [31]. The names of the plasmids used in the present study, their relevant characteristics and genetic changes in the 5′ T-*cyt* DNA, as described in detail by de Pater *et al.* [13, 14], are summarized in Fig. 1. Other relevant features of pAGS113 are that it confers bacterial resistance to tetracycline (2.5 mg/l) and that it has an artificial T-DNA with a plant kanamycin resistance gene. Transgenic potato plants were isolated either as described above for the transformations involving T-*cyt-uidA-nos*, or from shoot-forming stem galls as described earlier [32]. Resistance to kanamycin in the transformed shoots was verified as described above.

*Treatments and sampling of plant materials from T-*cyt-uidA-nos *transgenic lines*

From *in vitro* micropropagated shoots, five whole leaves were taken for analyses, roots were taken from several plants, bulk-harvested and subsampled. Stems consisted of internodal segments only

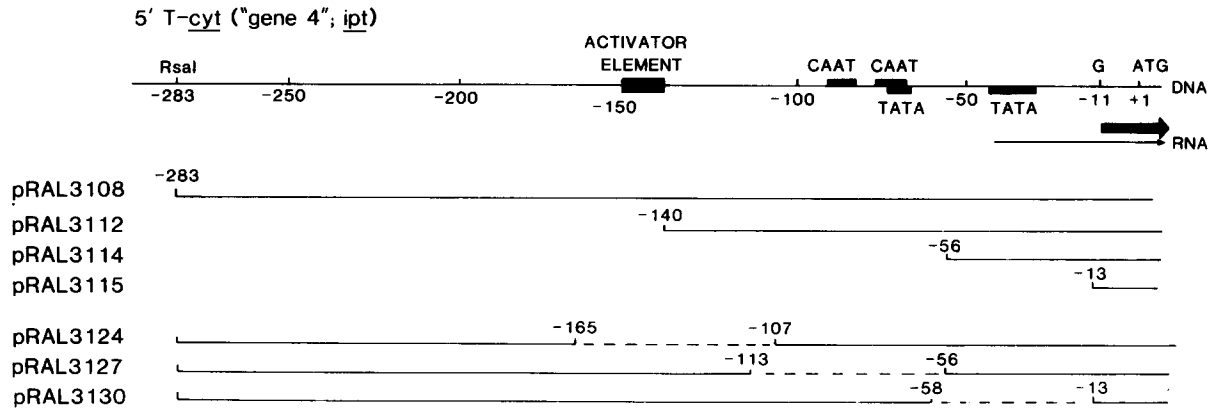


Fig. 1. Diagram of key features of the 5′ regulatory regions of the wild-type T-*cyt* gene (in pRAL3108) and a listing of plasmids with 5′ shortened (in pRAL3112, pRAL3114, pRAL3115) and mutated T-*cyt* genes. The top row gives the distance (in bp) between various sequences important in initiation of transcription relative to the first base in the ATG start with codon of translation. The plasmid numbers (left) correspond to plasmids constructed with particular 5′ T-*cyt* mutations as indicated and as described in detail elsewhere.

and in all cases sample weights ranged from 20–50 mg tissue fresh weight.

In vitro stem tumours were induced by infection of stem wounds with *A. tumefaciens* strain T37, grown on 1.8% agar solidified TY medium (5 g/l tryptone, 3 g/l yeast extract) at 29 °C. Each sample consisted of a number of tumours.

In vitro tubers were induced by placing nodal stem segments on solid MS medium supplemented with 80 g/l sucrose or with 60 g/l sucrose plus 1 mg/l benzylaminopurine (BAP). The segments were incubated in the dark at 24 ± 2 °C for periods of up to three months. Five samples were taken from five different tubers.

Soil-grown plants were established from *in vitro* grown shoots by transferring them to a controlled environment (CE) room and culturing in Eff-compost (Croxdon Horticultural Products, Stoke on Trent, UK) under 16 h day length with 21 °C day and 18 °C night temperature. Some plants were grown in soil from tubers harvested from soil-grown plants. Three or four young leaves were taken from well established soil-grown plants, they were cut into sections, mixed and then five subsamples were taken. Roots were bulk-harvested from a single plant and subsampled. Tuber samples were taken from five different tubers.

Determination of GUS activities

GUS activity in transgenic plants was determined either histochemically or by quantitative fluorometric analysis of tissue extracts using an automated plate reader (Fluoroskan II, Flowlabs, Rickmansworth, UK) [20, 21]. Fluorescence readings were standardized against 4-methyl umbelliferone (MU; Sigma No M-1508) dilutions in extraction buffer. GUS activities were related to tissue fresh weights (measured for excised organs and crown-gall tissues on a Mettler AE163 5-decimal balance) and to water-soluble extracted proteins, determined using a BioRad protein assay kit (BioRad No 500–0006). The average of two determinations per extract was taken and IgG was used as a protein standard.

In vitro *testing of wild-type and mutant* T-cyt *transgenic lines*

Lines transformed with wild-type and promoter-mutated T-*cyt* genes were examined for occurrence of characteristic T-*cyt*-related shoot phenotypes (absence or reduced growth of roots, callus formation at the base of the stems, formation of

multiple shoots from such callus and shoot branching, i.e. reduced apical dominance). Optimized *in vitro* culture conditions were used to determine shoot, crown and root growth of untransformed and transformed potato. In this quantitative growth assay, three replicated containers were used, with groups of ten plants each. The plastic containers were 10 cm high (Sterilin cat. no. 125 AP) and were filled with 75 ml (4.5 cm depth) MS20 medium solidified with 1.65 g/l Gelrite (Scott Lab). Growth was initiated from nodal stem segments with minimum amounts of internodal tissue from which leaves and petioles had been excised. After a standard 19-day culture period, the fresh weights of shoots, roots, and of the original stem segments plus whatever growth had occurred at the crown, the transition between shoots and roots, was measured per container and averaged for the three containers.

Tuberization of transformed and untransformed potato was compared by maintaining nodal cuttings in the dark at 23 ± 2 °C in glass containers with MS medium to which 80 g/l sucrose or 60 g/l sucrose plus 1 mg/l BAP was added. Tuber formation was scored by counting newly formed tubers at intervals during incubation periods of up to 30 weeks.

Results

Quantitation of GUS activity in transgenic plants

GUS activities were measured in extracts from tissues of nine transgenic lines, transformed with the chimaeric T-*cyt-uidA-nos* gene. The plants had been cultured *in vitro* and grown in soil in a CE room. The results of these measurements are summarized in Table 1 and Fig. 2. GUS activity is expressed on a protein basis but the results were similar if related to tissue fresh weights.

The measurements show that GUS activities differed between lines and between organs/tissues and showed variation for repeat analyses of one organ/tissue type of one line. An indication of the extent of variation for repeat experiments is given by the coefficients of variation (CV = 100 (SD/mean)) calculated from average values and standard deviations (Table 1). The CVs were smaller for some organs (roots and leaves from soil grown plants) than for other ones (tubers and leaves

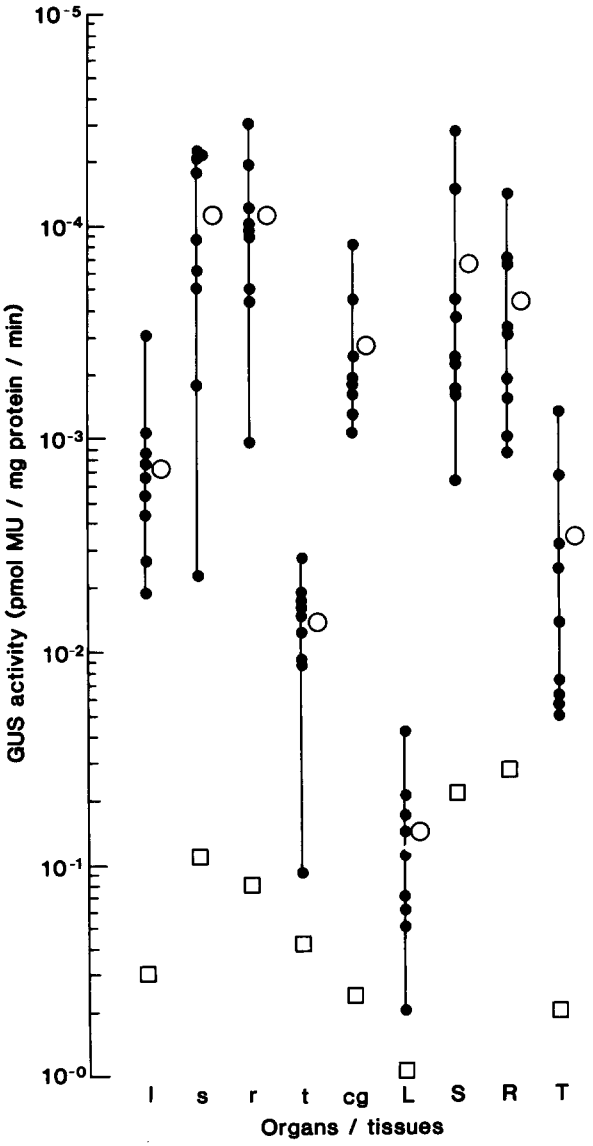


Fig. 2. Scatter plot of GUS activities in extracts of organs and tissues from potato shoots and plants of *S. tuberosum* cv. Désirée (□) and transgenic derivatives (○). Each value is calculated from five determinations as detailed in Table 1. The average value for a particular tissue/organ for all the transgenic lines is indicated by ○. Small lettering refers to organs and tissues from shoot cultures and capitals to soil-grown plants: l/L, leaves; s/S, stems; r/R, roots; t/T, tubers; cg, crown galls (shoot cultures only).

Table 1. Beta-glucuronidase (GUS) activities (pmol MU per mg protein per min) in extracts from organs and tissues from potato cv. Désirée and pTIP206-transformed derivatives. The values are averages (x) from five measurements and the corresponding coefficients of variation ($\sigma n{-}1/x$) are given in parenthesis (– means not tested). (T) refers to lines established from soil-grown tubers.

Line	Leaf	Stem	Root	Tuber	Crown gall
In vitro *grown plants*					
Désirée	3 (49)	11 (55)	8 (59)	4 (60)	2 (80)
DT206B3	2044 (42)	20068 (14)	9670 (12)	173 (77)	8105 (11)
DT206C1	575 (49)	233 (28)	980 (36)	271 (36)	–
DT206D2	676 (62)	20599 (31)	30045 (31)	146 (60)	2487 (37)
DT206E1	1099 (50)	8733 (52)	10360 (15)	87 (90)	1596 (117)
DT206F1	261 (110)	6120 (28)	5059 (17)	158 (50)	1932 (28)
DT206N2	439 (75)	5072 (65)	12009 (16)	90 (113)	4493 (49)
DT206O1	816 (82)	21010 (44)	19154 (34)	124 (70)	1084 (29)
DT206P2	554 (16)	17938 (17)	9057 (8)	168 (51)	1989 (18)
DT206Q2	178 (58)	1830 (78)	4425 (35)	9 (49)	1322 (37)
DT206C1 (T)	3607 (42)	–	–	–	–
DT206E1 (T)	2027 (74)	–	–	–	–
DT206O1 (T)	862 (50)	–	–	–	–
Soil-grown plants					
Désirée	1 (32)	22 (27)	28 (30)	2 (55)	
DT206B3	43 (8)	2273 (22)	1994 (10)	136 (13)	
DT206C1	21 (8)	3709 (32)	1042 (63)	244 (23)	
DT206D2	14 (12)	28270 (33)	14534 (8)	1372 (42)	
DT206E1	17 (24)	626 (23)	3358 (28)	72 (20)	
DT206F1	7 (10)	1766 (10)	865 (43)	61 (47)	
DT206N2	11 (23)	1679 (24)	1543 (15)	49 (44)	
DT206O1	6 (58)	15506 (69)	3167 (28)	314 (51)	
DT206P2	5 (22)	4603 (29)	7042 (12)	691 (33)	
DT206Q2	2 (8)	2234 (18)	6759 (8)	56 (58)	
DT206C1 (T)	6 (32)	–	–	–	
DT206E1 (T)	7 (25)	–	–	–	
DT206O1 (T)	8 (32)	–	–	–	

from *in vitro* grown plants). This difference may relate to the different sampling procedures followed (see Materials and methods). (Note that an average range for the CVs of 20–50% represents a two- to four-fold difference between maximum and minimum values for a set of five measurements.) A similar range of variation was found for the measurements of the protein content per fresh weight. This variation was largely caused by biological variation between tissues and/or differences between extractions since repeat assays of one extract normally gave results with coefficients of variation of less than 10%. Thus, the measurements not only gave average GUS activity values but also gave an indication of the precision of the comparisons of GUS levels between independently transformed lines and between the various organs/tissues both within and between the lines.

Comparing GUS levels between lines showed that some lines with relatively high (or low) GUS levels in one organ also had relatively high (or low) levels, respectively, in its other organs. Examples are DT206B3 and DT206C1 grown *in vitro*. However, contrasting results were obtained as well. For example, in DT206B3 the GUS activity in stems of *in vitro* plants was approximately nine-fold higher than in stems of soil-grown plants, whereas in DT206C1 approximately sixteen-fold lower levels were detected in the stems of the *in vitro* grown plants.

Grouping the results for all the plants and organs/tissues as a scatter diagram (Fig. 2)

showed that the highest average GUS activities were in the roots and stems of *in vitro* grown plants (*ca.* 11000 GUS activity units per pmol MU per mg protein per min) and activity decreased across organs in the following order: crown galls, roots and stems of soil-grown plants, leaves of *in vitro* grown plants, tubers from plants in soil and *in vitro* and leaves of soil-grown plants. GUS activities in the latter were at or near background, i.e. those measured for untransformed Désirée plants (Table 1). The range of GUS activities was nearly a hundred-fold for some organs and tissues (e.g. stems) and less than ten-fold for others (e.g. crown galls); (Fig. 2).

Changing culture conditions and continuous subculturing

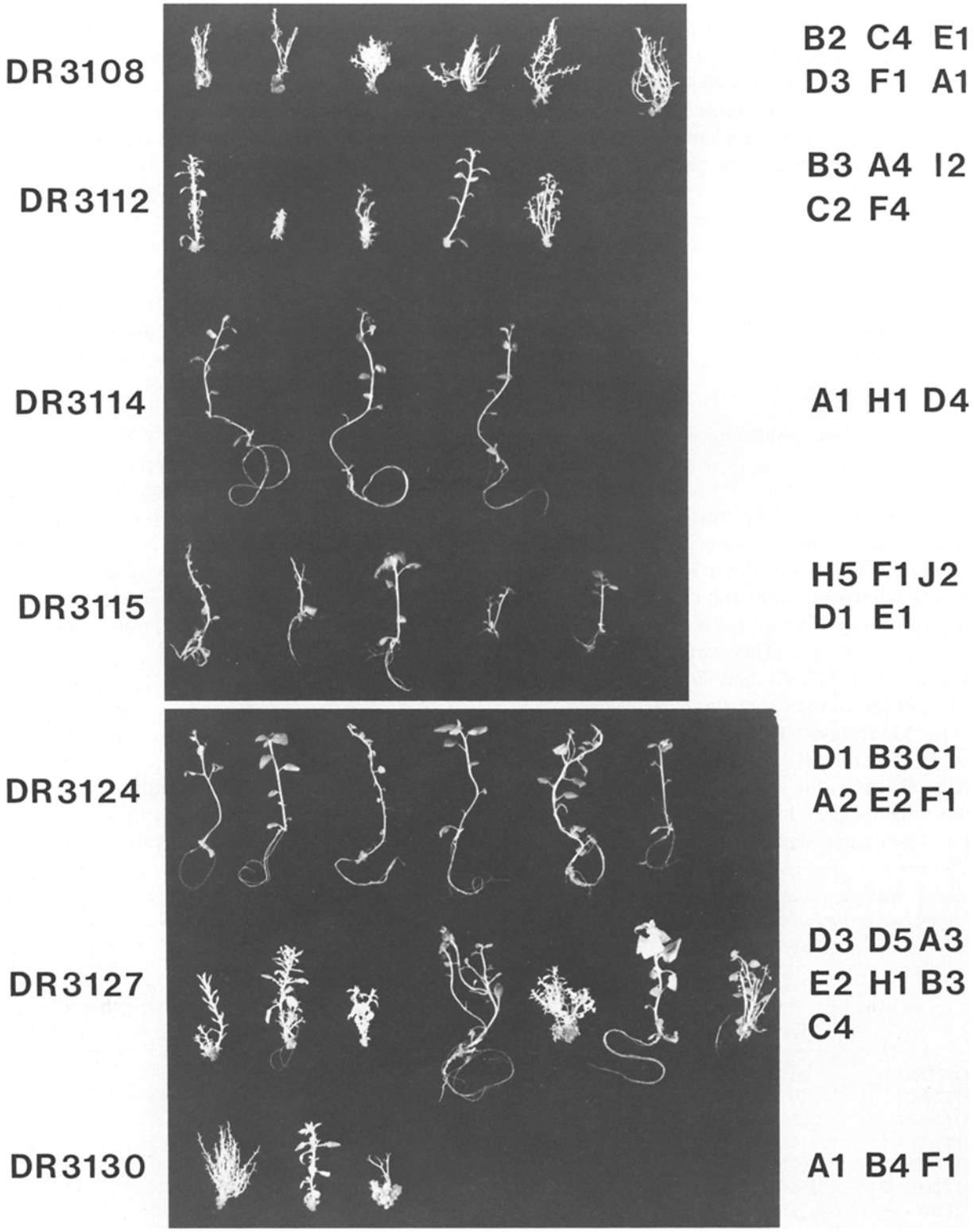
The reversibility of the low GUS activities in leaves of soil-grown plants was examined by comparing GUS activities in leaves of soil-grown plants established from soil-derived tubers, with those in leaves of shoot cultures established from the same tubers. This was done for lines DT206C1, DT206E1 and DT206O1 (Table 1). The results showed that the transfer back to *in vitro* culture re-established the higher leaf GUS levels (Table 1). It was noted, however, that for line DT206C1, the GUS-levels for leaves *in vitro* were significantly higher (approximately 6-fold) for the cultures established from tubers than in the original shoot cultures (Table 1). Similar changes in the average GUS activities in the leaves of the shoot cultures, were also observed by simply maintaining the shoot cultures *in vitro* for a length of time. In an earlier set of measurements, carried out approximately eighteen months prior to the measurements in Table 1, we had determined GUS activities in eight of the transgenic lines. These measurements for GUS levels in roots, stems and leaves of *in vitro* grown shoots were made on a fresh weight basis. Comparing the two sets of results (Table 2) showed only small changes in GUS activities for most root/stem measurements. In some cases, however, there were considerable differences such as the significant reductions in all organs for DT206C1 and increases in stems and roots only for DT206N2. In contrast, in most lines there were reductions in GUS levels in the leaves of the *in vitro* grown plants (Table 2). One implication of this result is that the GUS activities in the leaves of *in vitro* grown plants, as summarized in Table 1, represent an underestimate of the levels in plants established shortly after transformation/regeneration.

Histochemistry

Attempts to identify, by histochemical analysis, in which cells GUS activities were most abundant were only of limited value. Generally, the staining

Table 2. Beta-glucuronidase (GUS) activities (pmol MU per mg fresh weight per min) in extracts from organs from pTIP-transformed derivatives of potato cv. Désirée. The materials were harvested from shoot cultures established early after isolation (early) and following an 18-month period of micropropagation (late). The values have been calculated as for Table 1.

Line	Leaf		Stem		Root	
	Early	Late	Early	Late	Early	Late
DT206B3	337 (38)	100 (33)	230 (47)	205 (32)	200 (44)	123 (8)
DT206C1	105 (45)	36 (38)	90 (35)	2 (20)	111 (25)	9 (37)
DT206E1	259 (30)	36 (32)	60 (79)	52 (31)	86 (41)	125 (30)
DT206F1	32 (41)	11 (100)	30 (41)	42 (28)	35 (45)	35 (14)
DT206N2	21 (55)	12 (54)	4 (99)	21 (55)	20 (28)	58 (29)
DT206O1	64 (52)	40 (48)	138 (29)	126 (47)	109 (58)	137 (29)
DT206P2	25 (21)	31 (35)	148 (22)	217 (29)	60 (40)	108 (31)
DT206Q2	5 (28)	12 (55)	12 (19)	23 (58)	25 (42)	77 (38)


DR3108
B2 C4 E1
D3 F1 A1
DR3112
B3 A4 I2
C2 F4
DR3114
A1 H1 D4
DR3115
H5 F1 J2
D1 E1
DR3124
D1 B3C1
A2 E2 F1
DR3127
D3 D5 A3
E2 H1 B3
C4
DR3130
A1 B4 F1

of *in vitro* leaves, stems and roots showed similar patterns as seen for transgenic tobacco plants transformed with a CaMV35S-*uidA-nos* chimaeric gene [20] with most activity in vascular areas in particular in phloem cells and in root tips. The results were also very variable even for organs on the same plant. Although staining could be associated in some areas with damaged tissue, no clear evidence of wound induction of GUS activity was seen in the *in vitro* grown plants.

*Transformation of potato with a wild-type T-*cyt *gene and six promoter-mutated derivatives*

For comparison with the analysis of the T-*cyt-uidA-nos* gene in transgenic cv. Désirée plants, seven *A. tumefaciens* strains were used to obtain over forty correspondingly transformed potato lines, each transformed with a wild-type T-*cyt* gene, which includes all its known regulatory DNA important in gene expression (pRAL3108), or with one of six promoter-mutated T-*cyt* genes (Fig. 1), which have known variation in their tumour-forming abilities on test plants tobacco, tomato and *Kalanchoe*. Typically, all isolated kanamycin-resistant shoots that were transformed with the wild-type T-*cyt* gene showed little distinct phenotypic variation between shoots cultured *in vitro*; this was both for the individual shoots of one line and the shoots of different lines. They all showed callus formation at the base of their stems, generally showed no or very poor root formation and formed short and frequently branching multiple shoots from their callus base (Fig. 3). As expected, they were like potato plants transformed with T-DNA from shoot-inducing *A. tumefaciens* strains isolated previously [32]. In an analogous situation, kanamycin-resistant plants regenerated from infections using pRAL3114, pRAL3115 and pRAL3124 were relatively normal and also showed relatively little phenotypic variation for shoot growth *in vitro*, both between the shoots of one line and between the lines (Fig. 3). The widest spectrum of variation in shoot phenotype between lines was seen from infections using pRAL3112 and pRAL3127. Both the above types of phenotypes were present, as well as intermediate types (Fig. 3). For unknown reasons, only three lines were successfully isolated using pRAL3130 and these resembled the pRAL3108 transformants (Fig. 3). These plants, however, proved particularly unamenable to grafting, even *in vitro*, and attempts at growing them in soil were consistently unsuccessful.

*T-*cyt-*transformed potato* in vitro

Following the establishment of the *in vitro* shoot cultures, the acquired changes in growth and development were examined not only phenotypically, but also more quantitatively by culturing the shoots under two particular *in vitro* test conditions. Firstly, plant growth was quantified by measuring fresh weight increases over a constant growth period for replicated populations of shoots grown under optimized *in vitro* culture conditions (see Materials and methods). Secondly, the timing of induction of tuberization under standard *in vitro* conditions was determined. It can be seen from Table 3 that the *in vitro* growth test showed clear differences between the lines, in particular in their root growth. Observed differences in crown weights were small and probably largely reflected differences in the weights of the cuttings from which the plants were grown. Furthermore, only those lines with severely inhibited root growth showed clearly reduced shoot growth too (Table 3). The (near) normal phenotypes of shoots transformed using plasmids pRAL3114, pRAL3115 and pRAL3124 was confirmed by

Fig. 3. Potato shoots transformed with T-*cyt* genes. The shoots were isolated from infections of potato cv. Désiré with agrobacteria carrying various pRAL plasmids (see Fig. 1). This resulted in the isolation of correspondingly transformed lines (e.g. DR3108 from plants transformed with pRAL3108) with further specification of the particular transformation event (A, B, etc) and the particular shoot taken from the regeneration (1, 2, etc.) to give single shoot-derived lines (e.g. DR3108A1), used for subsequent micropropagation.

Table 3. The fresh weights of shoots, crowns and roots of shoot cultures of potato cv. Désirée and a number of its transgenic derivatives after propagation *in vitro* for 19 days. The mean fresh weights and standard derivations (SD) for each line were calculated from measurements on populations of 10 plants, each replicated three times. The results for DR3124A2 were from measurements on three populations of six plants only, multiplied by 1.7. No replications were done for the measurements on Désirée shoot cultures grown on media with zeatin.

Line	Fresh weight (mg)		
	Shoot	Crown	Root
Désirée	1044 ± 131	139 ± 17	238 ± 35
DR3108A1	200 ± 69	91 ± 33	18 ± 13
DR3112C2	611 ± 181	238 ± 40	32 ± 26
DR3114H1	918 ± 162	254 ± 7	61 ± 37
DR3115H5	798 ± 34	207 ± 33	205 ± 204
DR3115J2	1196 ± 56	255 ± 22	294 ± 6
DR3124A2	772 ± 207	181 ± 13	298 ± 81
DR3124B3	1057 ± 134	192 ± 16	327 ± 41
DR3124C1	926 ± 175	210 ± 16	286 ± 30
DR3127B3	1087 ± 175	235 ± 14	225 ± 78
DR3127C4	795 ± 108	103 ± 20	104 ± 24
DR3127D5	936 ± 275	261 ± 103	21 ± 15
Des + 0.01 zeatin	905	212	216
Des + 0.1 zeatin	392	247	16
Des + 1.0 zeatin	55	172	–

their similar plant growth as the Désirée shoot cultures with the exception of DR3114H1 which had reduced root growth. Of the pRAL3127-transformed lines, the morphologically normal DR127B3 also showed normal increases in plant weight during the standard 19-day growth period, but clear phenotypic diversity between shoots of the other independently transformed pRAL3127 lines was reflected by their widely differing amount of plant growth (Table 1). This ranged from normal to completely lacking root growth in DR3127H1 (not shown). The result contrasted with pRAL3108 transformations where only DR3108A1 showed any sign of some root growth during previous subculturing on standard tissue culture media; DR3108A1 had measurable, but very diminished, root growth as well as reduced shoot growth.

For comparison, three treatments were included in which Désirée shoots were grown on media to which 0.01, 0.1 and 1 mg/l zeatin was added (Table 3). This showed no significant inhibition at the lowest concentration tested, considerable inhibition at the intermediate concentration and almost total inhibition, even of shoot growth, at the highest zeatin concentration.

Table 4 shows the results of *in vitro* tuberizations. All morphologically normal lines, including three cultivars other than Désirée (Estima, Record and Maris Bard), and two transgenic lines (DR3127A2 and DR3124A2), had similar normal *in vitro* tuberization properties. The phenotypically contrasting lines DR3108E1, DR3108A1, DR3127D5, DR3127H1 and Mb1501B, however, all gave no or few tubers even after prolonged incubation periods.

Discussion

T-cyt-uidA-nos *gene expression*

The analysis of potato plants transformed with a chimaeric T-*cyt-uidA-nos* gene showed strong dependence of the expression of the gene on plant growth conditions. The reduction of GUS levels in leaves of soil-grown plants compared with *in vitro* grown plants contrasts with, for example, a similar analysis in our laboratory of transgenic potato plants transformed with a CaMV35S-*uidA-nos* gene (manuscript in preparation). These had high average GUS levels in all tissues, including leaves, from shoot culture plants and soil-grown plants. The CaMV35S-*uidA-nos* transgenic potato lines, like the current lines, also had somewhat lower GUS activity levels in the *in vitro* and soil-grown tubers (manuscript in preparation). These observations strongly suggest that initiation of transcription from the T-*cyt* promoter is much reduced, specifically in the leaves of soil-grown plants. However, the conclusion must be qualified in several ways. For example, the current analysis was in potato only and hence the generality of the observations remains to be examined. It is also clear that the experiments do not clarify which particular aspect of the environmental change or indeed by which molecular

Table 4. Summary of *in vitro* tuberization tests on untransformed potato shoots and T-*cyt* transformed derivatives. The names of the lines tested are given in the first column. In Experiment 1, nine nodal stem segments were tested per line on MS80 medium. In Experiment 2, four nodal stem segments were tested per line on MS60 plus added BAP. In Experiment 3, 30 nodal stem segments were tested also on MS60 plus BAP. W4, W22, etc. indicate the number of weeks of dark incubation before the total numbers of tubers formed were counted. NT, no tested.

Line	Exp. 1		Exp. 2		Exp. 3			
	W4	W22	W2	W4	W22	W2	W3	W17
Désirée	–	13	1	3	6	7	20	24
DR3108A1	–	1	–	–	–	NT		
DR3108E1	–	1	–	–	–	NT		
DR3124A2	–	11	–	1	8	1	12	24
DR3127A2	1	11	4	4	5	5	16	27
DR3127D5	–	–	–	–	–	NT		
DR3127H1	–	–	–	–	–	NT		
DR3130F1	–	–	–	–	–	NT		
Maris Bard	1	10	1	1	3	2	13	19
Estima	–	6	3	3	6	NT		
Record	–	4	–	–	6	NT		
Mb1501B	NT		NT	NT		–	–	22

mechanism the change in plant culture conditions causes the presumed change in T-*cyt* transcription initiation. A possible clue to the mechanism comes from the analysis of the dyad G-box of the *Arabidopsis Adh* gene [27], which has homology with the T-*cyt Activator* element. *In vivo* and *in vitro* footprinting has shown that although proteins with specific binding activity for the G-box were present in extracts of both cell cultures and leaves, *in vivo* binding to the G-box occurs in the cell cultures only. Differences in *in vivo* binding activity could be associated with specific covalent modifications to the G-box binding factor(s), such as protein phosphorylation [27].

Biological implications

Irrespective of the molecular mechanism that underlies the silencing of the T-*cyt-uidA-nos* gene expression specifically in the leaves of soil-grown potato plants, the observation has a number of biological implications. Firstly, it shows that the very considerable down-regulation in leaves of the initiation of transcription from the T-*cyt* promoter, caused by the change in environment, is probably the single most important factor in the phenotypic normalization that occurs when T-*cyt* transformed potato plants are transferred from *in vitro* to soil in a controlled environment [32, 33, 34]. It must be mentioned, however, that the current analysis does not exclude that other types of regulation on the accumulation of T-*cyt* gene expression products could occur too, such as regulation directly on T-*cyt* mRNA and protein levels or on cytokinin metabolism, perhaps in certain cell types only and perhaps influenced by plant culture conditions. Of further importance to the phenotypic normalization appears the variation in relative and absolute GUS levels in organs and tissues of the different T-*cyt-uidA-nos* transformed plants. These differences are probably caused by genomic position effects which are commonly found in transgenic plants [11]. The result suggests that the occurrence of variation in T-*cyt* gene expression in crown gall cells from position effects, provides an important possible explanation for the difference between independently isolated crown gall lines in growth properties, in capacity to regenerate shoots and, indeed, in amenability to be grown in a controlled environment room [34, 35, 39]. It is noted however, that there are well documented examples of other observations, too, that may be important in explain-

ing the phenotypic differences such as T-DNA methylation [2, 23, 35] and acquisition of gross chromosomal abnormalities [28, 32].

A second implication of the reduced expression of the T-*cyt-uidA-nos* gene specifically in leaves of soil-grown plants, is that initiation of expression in other cells appears to remain high. If this were also the case for the T-*cyt* gene, then this would be of potential significance in relation to the above mentioned possible controls on T-*cyt* RNA or protein accumulation in the other cells and in relation to questions on the biological significance of differences in levels of cytokinins, and of sensitivity to cytokinins in different cell types [9, 10, 26, 30, 34, 38].

A third biological implication of our results is that suppression of T-*cyt* gene expression in potato leaves is a possible determinant in crown gall formation. It would be a fundamental cause for the generally observed poor susceptibility of wounded potato leaves, growing on plants in soil, to infection by virulent *Agrobacterium* strains.

Finally, the difference in absolute GUS activity levels between the transgenic potato lines and in the ratios of GUS activities for the various organs/tissues, not only agrees with the commonly observed variation in transgene expression between independently isolated transgenic plants [11], but in the case of natural *Agrobacterium* transformation it will have particular biological relevance. It creates genetic diversity amongst independently transformed cells upon which selection can operate to reach optimal growth of crown galls as appropriate to the particular type of transformed cells and the particular environmental conditions.

*T-*cyt *promoter mutations and genomic position effects*

Transformation in potato cv. Désirée using the wild-type T-*cyt* gene and six promoter-mutated derivatives gave shoots that differed widely in phenotype. When we look at the total population of transformed shoots, the phenotypes varied from normal, such as seen consistently from transformations using pRAL3114, pRAL3115 and pRAL3124, to those with characteristic T-*cyt*-related phenotypes, such as from transformations using pRAL3108, and the extreme phenotype shoots, with no or poor growth, that came from the pRAL3130 transformations. These results have implications for the function of the various deleted DNA regions. They confirmed the absolute requirement for crown gall formation on all plants tested of the 150 bp *Activator* element [14, 15] and extended it as an essential requirement for T-*cyt* related phenotypes in regenerated shoots. The phenotypes of the pRAL3130 transformed potato lines, and the difficulty experienced in isolating them, suggest that the deletion in the T-*cyt* promoter in pRAL3130 (position −58 to −13, including one TATA region) introduces an altered control upon T-*cyt* gene expression which makes it less likely that transformed cells can be grown into shoots and that the shoots can be grown into plants. (Note that in tumour induction assays pRAL3130 induces normal tumour formation under standard test conditions [14, 15].) Of particular interest are the transformations using pRAL3127. Like pRAL3130, the plasmid is indistinguishable from the control plasmid, pRAL3108, in standard tumour induction assays, but the composition of the phenotypically heterogeneous population of pRAL3127-transformed potato shoots contrasts considerably with the phenotypically homogenous population of pRAL3108-transformed potato shoots. The phenotypes of the pRAL3127 shoots varied widely from (near) normal to typically T-*cyt*-transformed. The result shows that the deletion of the T-*cyt* promoter region −113/ −56, which includes putative CAAT regions [14, 15], is important in establishing the difference between the populations of the pRAL3127- and pRAL3108-transformed plants. It is possible that the deleted DNA is important in determining a reduced average level of initiation of T-*cyt* expression throughout the population of transformed plants. This can be achieved either by some reduction in the initiation of T-*cyt* gene expression in all the independently isolated lines that make up the population or by causing very low T-*cyt* gene ex-

pression in a few lines only. This would effectively give a wider range of expression levels within the population. The observed results contrast further with the results of transformations in tobacco using pRAL3108 [13] or other shoot-inducing strains such as *A. tumefaciens* T37 [39]. Isolated transgenic tobacco shoots varied widely in phenotype and hence the population appeared phenotypically heterogenous, like in potato for the pRAL3127-transformed shoots. It is not unusual to see contrasting plant populations where one set of genes behaved differently in two different genetic backgrounds. For example, Ri T-DNA transformation in potato cv. Désirée and cv. Bintje gave populations of transgenic plants that were altered homogenously and heterogeneously, respectively [19]. Also in other gene expression studies using transgenic plants, differences have been reported in the type and range of expression levels of the introduced genes between independently isolated plants. In a few studies there were narrow-range differences [8, 40], but more commonly the ranges are wider [e.g. 36]. How the mutations in the T-*cyt* TATA and CAAT regions relate to induction of gene expression in a population of independently transformed lines can be tested directly by chimaeric gene analysis in transgenic plants and a comparison with the results in the present study.

Conclusions

In conclusion, examination of GUS levels in transgenic potato plants transformed with a chimaeric T-*cyt-uidA-nos* gene has shown that its expression is under the control of plant culture conditions, being silenced specifically in leaves of plants grown in soil in a controlled environment room. Expression of the T-*cyt-uidA-nos* gene was also specifically suppressed to some extent in leaves of *in vitro* plants by simply maintaining the lines by micropropagation over a period of time. These observations were compared with the results of other studies on the structure and expression of the wild type T-*cyt* gene, and on the phenotypes of independently isolated, T-*cyt*-transformed potato shoots and the compositions of populations of plants, transformed with particular T-*cyt* genes. The comparison extends our insight into the molecular and cellular mechanisms that underlie crown gall formation and into the causes of the diversity in growth properties of crown gall lines and plants regenerated from them. Specific DNA sequences in the T-*cyt* promoter region may be important in determining variation between populations of transformed plants in gene expression in independently transformed cells and the cell lines and plants established from them. Finally because of DNA homology between the T-*cyt Activator* sequences and G-box sequences, both with demonstrated importance in gene expression, and because these or similar sequences are common in 5′ flanking DNA of a variety of plant genes, the current findings are potentially relevant to the study of control of gene expression for many plant genes, in particular in relation to differing plant culture environments.

Acknowledgements

We thank the Agricultural Genetics Company, Cambridge, and the Department of Scientific and Industrial Research, New Zealand, for support. The work is also supported by a joint EC grant (BAP Grant 0099-UK) with the Leiden University and discussions and exchange of materials with the members of its Department of Plant Molecular Biology are appreciated. We thank Brian Forde for critically reading the manuscript. This work was carried out under licence of the Plant Health Division of the Ministry of Agriculture, Fisheries and Food.

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