A transcription activation system for regulated gene expression in transgenic plants

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ABSTRACT A widely applicable promoter system is described that allows a gene of interest to be activated in specific plant tissues after a cross between defined transgenic lines. The promoter, pOp, consists of lac operators cloned upstream of a minimal promoter. No expression was detected from this promoter when placed upstream of a β-glucuronidase (GUS) reporter gene in transgenic plants. Transcription from the promoter was activated by crossing reporter plants with activator lines that expressed a chimeric transcription factor, LhG4. This factor comprised transcription-activation domain-II from Gal4 of *Saccharomyces cerevisiae* fused to a mutant lac-repressor that binds its operator with increased affinity. When LhG4 was expressed from the CaMV 35S promoter, the spatial and quantitative expression characteristics of the 35S promoter were exhibited by the GUS reporter. The LhG4/pOp system may be used to study toxic or deleterious gene products, to coordinate the expression of multiple gene products, to restrict transgene phenotypes to the F1 generation, and to generate hybrid seed. The LhG4 system offers spatially regulated gene expression in the tissues of whole plants growing under normal conditions without the need for external intervention. It complements inducible expression systems that offer temporal control of gene expression in tissues that can be treated with inducing chemicals.

Plant science, both basic and applied, relies increasingly on the use of transgenes to manipulate biological processes in transgenic plants. Thus antisense RNA, dominant negative mutants, dominant gain-of-function mutants, ectopic expression, and overexpression have provided powerful tools to investigate a broad range of biochemical pathways. However, some of these molecular genetic approaches are not easily applied to genes that control fundamental processes of plant growth, differentiation, and reproduction. This is primarily because manipulating such critical genes can be severely detrimental to plant growth and survival and so preclude the generation and propagation of useful transgenic plants.

One solution has been to use “tissue-specific” promoters to restrict the activity of transgenes to certain tissues (1, 2). This approach is far from satisfactory as many such promoters are active during the process of regenerating transgenic plants. Tissue specific expression also restricts the scope of the analysis to a few cell types and does not always allow the appropriate tissues to be studied. Another option is the use of heat-shock promoters that can achieve relatively high level expression in many cell types (3). This has the disadvantage that the subsequent analysis is performed on plants subjected to heat stress, growth conditions must be carefully controlled, and problems have been encountered with expression of genes in plants not induced by heat shock (3–5).

To overcome these limitations several attempts have been made to develop chemically regulated gene expression systems (6–10). Again, these are not entirely satisfactory as they are either relatively inefficient (high background and/or only modest induction), dependent on sustained gene repression, not applicable to commonly studied plant species, or reliant on the application of chemicals at concentrations that may be toxic to plants. The best characterized system is a tetracycline inducible promoter developed for tobacco (8, 11–13). This chemical induction system provides useful temporal control of gene expression in tobacco cells though its usefulness and reliability for spatial control of gene expression in the various tissues of whole plants is less clear. Also it appears that this system is not effective in *Arabidopsis* (14).

It is necessary to establish a generally applicable system for effective spatial control of transgene expression that does not require external intervention or imposition of environmental stress. Here we report a system based on chimeric transcription activators and promoters. In essence, “reporter plants” are generated with the gene of interest cloned downstream of a promoter that comprises simply a TATA-box and binding sites for a transcription factor that is not present in plants (Fig. 1A and B). Consequently, the gene of interest is unlikely to be expressed when introduced into wild-type plant cells. This allows phenotypically normal transgenic lines to be generated and propagated without interference from the transgene or selection against its expression. Expression of the transgene is achieved by crossing these reporter plants with an “activator plant” that expresses a novel transcription factor that specifically recognizes the binding sites in the transgene promoter (Fig. 1A and B). In this way the gene of interest will be expressed only in those cells of the progeny in which the transcription factor is expressed, and this will depend in turn on the promoter selected to express the transcription factor. Thus the effects of the transgene can be studied in the progeny of crosses between activator and reporter lines.

RESULTS

To construct a stringent regulatory system based on promoter activation we sought to use transcription factors with sequence-specific DNA-binding activities that were not normally found in plants. Initially we selected the *Saccharomyces cerevisiae* Gal4 protein that had been used successfully in an analogous regulatory system for *Drosophila* (15) and shown to act as a transcription factor in transient expression experiments with tobacco (16). Subsequently we developed an alternative system based on a modified *Escherichia coli* lac-repressor. Here we describe only the lac-repressor system as

Abbreviation: CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; 4-MU, 4-methyl-umbelliferyl...
fusion, LhG4, was inserted between a CaMV 35S promoter and polyadenylation signal in a binary Ti-plasmid vector to generate pLh-G4 (Fig. 1C). For comparison, a lac repressor derivative, LacI-VP16, that had been optimized for use as a transcription activator in mammals (20), was cloned in the same binary vector, to generate pL-VP16. This construct differed considerably from pLh-G4 as it had a wild-type lac repressor DNA-binding domain, a simian virus 40 large T-antigen nuclear-targeting signal, and the herpes simplex virus VP16 activation domain. pLh-0, which expresses the lacHis coding sequence without a transcription activation domain served as a negative control.

To construct the reporter plasmid pOp-GUS, a glucuronidase (GUS) reporter gene was cloned downstream of a minimal CaMV 35S promoter (+3 to –50 relative to the transcription start site) and two copies of an optimized lac operator were inserted upstream (operator 310 of ref. 21) (Fig. 1C). To reduce the risk of the minimal CaMV 35S promoter being activated in cis by promoter sequences in the T-DNA or neighboring plant DNA, the minimal promoter in pOp-GUS was placed at least 2kb from the left and right T-DNA border sequences, and 2.5 kb away from the only fully functional plant promoter on the T-DNA, the relatively weak (22) nopaline synthase promoter. Plasmid pTA-GUS was identical to pOp-GUS except that it lacked the operator sequences.

LhG4 Is Functional in Plants and Their Progeny. Transient expression experiments in tobacco protoplasts with pLh-G4 (activator), pOp-GUS (reporter), and the control constructs provided initial evidence for activation of reporter gene expression by LhG4 (data not shown). This activation was found to be dependent on the presence of both lac operator sequences in the reporter construct and the Gal4 activation-domain in the activator construct, so we proceeded to test activator and reporter constructs in stable transfectants. Leaf samples from 10 independent rooted primary transfectants generated with reporter plasmid pOp-GUS were screened for GUS activity using the histochemical assay, and none showed detectable activity even in prolonged (16 hr) assays. This confirmed that the GUS reporter construct was not efficiently activated in cis from the T-DNA or flanking chromosomal DNA in these plants and that, as expected, tobacco cells do not have a transcriptional activator that can recognize the lac operator sequences used here.

Six pOp-GUS transfectants (16/1, 16/3, 16/12, 16/15, 16/51, and 16/56) were selected as reporter plants for retransformation with the activator plasmids pLh-G4 and pL-VP16. Retransformants, selected by their resistance to methotrexate, were screened for GUS activity by histochemical assay of leaf pieces.

Of 40 independent reporter plants retransformed with pLh-G4, 10 showed intense staining similar to or stronger than 35S-GUS plants, two exhibited intermediate staining and two more showed weak staining. The distribution of GUS-expressing retransformants among the individual reporter plants appeared to be nonrandom. Thus strong or intermediate staining was observed in 5 of 12 retransformants derived from reporter 16/12 and in 7 of 8 retransformants derived from reporter 16/56, but not in any of the 20 retransformants regenerated from the remaining four reporter plants (16/1, 16/3, 16/15, and 16/51). This probably reflects the influence of the integration site and flanking plant DNA on the expression of the reporter gene in each reporter line, and suggests that in a favorable location the reporter may be effectively activated by most activator loci.

Of 19 independent reporter plants retransformed with pL-VP16 (which encodes LacI-VP16), one showed intermediate staining and a further five showed weak staining, suggesting that pL-VP16 is a less effective activator construct than pLh-G4. This suggestion was supported by analysis of GUS transcript abundance in 16/56 retransformants (Fig. 2). Five
pLh-G4 retransformants that had shown strong GUS activity were all found to express both GUS and LhG4 transcripts of the expected size. In a sixth retransformant, line 16/56-G3 in which no GUS staining or transcript was observed, the only detectable LhG4-homologous transcript was much larger than expected. In marked contrast to the pLh-G4 retransformants, GUS transcripts were barely detectable or undetectable in any of the pL-VP16 retransformants, even though LacI-VP16 activator transcripts were generally far more abundant than LhG4 transcripts. As LacI-VP16 differs in several respects from LhG4 (see above) there are a number of possible explanations for its apparently poorer activity and greater transcript abundance. At this stage we conclude only that pL-VP16 is a less effective activator construct than pLh-G4.

The cauliflower mosaic virus (CaMV) 35S-promoter is reported to be more active in the older leaves than in younger ones (23). To test whether this is reflected by the GUS reporter when its expression is directed by LhG4 under CaMV 35S control, extractable GUS activity was measured in 6 successive leaves on each of 5 pLh-G4 retransformants derived from reporter plant 16/12. There was a progressive increase in expression levels from an average of 3.2 nmol 4-methyl-reporter plant 16 leaves on each of 5 pLh-G4 retransformants derived from control, extractable GUS activity was measured in 6 successive pL-VP16 is a less effective activator construct than pLh-G4. LhG4 transcripts. As LacI-VP16 differs in several respects of the pL-VP16 retransformants, even though LacI-VP16 activator transcripts were generally far more abundant than LhG4 transcripts. As LacI-VP16 differs in several respects from LhG4 (see above) there are a number of possible explanations for its apparently poorer activity and greater transcript abundance. At this stage we conclude only that pL-VP16 is a less effective activator construct than pLh-G4.

The GUS Reporter Is Expressed After Crossing Activator and Reporter Plants. The aim of this work was to generate a system that would allow gene expression to be activated by crossing reporter and activator plants. To test whether this was possible with the pOp/LhG4 system, activator plants were generated by transforming tobacco with pLh-G4. Three such activator plants (10/1–2, 10/1–6, and 47/1–21) were chosen at random and each was crossed with the reporter plants 16/12 and 16/56. The same three activator plants were also crossed with a control plant, 13/4, that had been transformed with pTA-GUS that contains a GUS reporter gene driven by the minimal CaMV 35S-promoter but without lac operators upstream. The frequencies of hygromycin and methotrexate resistant F1 seedlings suggested that each activator plant contained a T-DNA at a single locus and confirmed that the reporter plants had T-DNAs at 4 or more loci. F1 seedlings resistant to both hygromycin and methotrexate were assayed histochemically for GUS activity. This demonstrated that all three randomly selected activators were able to induce GUS expression in the F1 progeny of both reporter plants but not in the progeny of the negative control plant 13/4 (Fig. 4). Activator plant 10/1–6 was able to activate GUS expression to 35S-GUS levels in all seedling organs after crossing with either reporter plant. In contrast, GUS expression induced by activator 47/1–21 was weaker and was restricted principally to cotyledons and hypocotyls. In subsequent experiments, the GUS reporter construct was found also to be expressed as expected in the F2 progeny of 10 randomly selected F1 plants derived from a cross between reporter 16/56 and activator 10/1–6 (data not shown). Thus the pOp/LhG4 system was active in three successive generations of transgenic plants.

DISCUSSION

We have established a promoter and transcription factor system that allows a gene of interest to be expressed only after crossing of reporter and activator plants. This system may be useful (i) if the gene of interest is expected to interfere with regeneration or propagation of transgenic material, (ii) if its activity is required only in the progeny of a cross, or (iii) if it must be expressed in several different specific tissues. It may also allow the expression of several transgenes to be regulated respectively, and this may account for the low activity in some samples.

Seedlings of the T2 generation obtained by selfing the pOp-GUS/pLh-G4 retransformants were analyzed for methotrexate and hygromycin resistance. This suggested that the activator T-DNA was present in each line at either one or two Mendelian loci, whilst the reporter T-DNA appeared to be present in each line at four or more loci. Histochemical analysis of GUS activity in T2 seedlings showed that the reporter gene could be activated in all the major organs (Fig. 3A) with staining intensities similar to those of 35S-GUS seedlings. The GUS activity measured in extracts of entire T2 seedlings (average 12 nmol·4-MU·min⁻¹·mg protein⁻¹) was also comparable to that of homozygous 35S-GUS seedlings and exhibited relatively little variation (Fig. 3B) despite the presence of multiple T-DNA insertions. Similarly, when GUS expression was assayed histochemically, relatively little variation in its expression pattern was observed in eight populations of 15 T2 seedlings derived from eight independent retransformants (data not shown). Thus the pOp/LhG4 system appeared to be heritable and fully functional in a subsequent generation.

Extractable GUS activity measured in T2 seedlings derived from the 16/12 and 16/56 reporter plants was indistinguishable from that of untransformed plants (<0.01 nmol·4-MU·min⁻¹·mg protein⁻¹), indicating that the reporter was effectively silent in all organs of the seedling and that GUS activity can increase by 3 orders of magnitude in the presence of pLhG4.
coordinately without multiplication of plant promoter sequences.

The promoter comprises a minimal promoter fragment of the CaMV 35S-promoter with two ideal (24) lac operator sequences cloned upstream. The transcription factor, LhG4, consists of a high affinity DNA-binding mutant of the E. coli lac repressor (21) fused to a transcription activation domain from the yeast Gal4 protein.

We envisage that this promoter may be used to introduce a gene of interest into tobacco where it will initially be silent. In this way transgenic “reporter” plants may be regenerated without interference from the transgene. This would be advantageous when the gene of interest is expected to interfere with some essential metabolic or cellular function or when its activity is required in the F1 generation only. The use of constitutive promoters in such cases is likely to lead to the recovery of plants in which transgene expression is weak and variable owing to position effects and gene silencing phenomena. In the pOp/LhG4 system described here, the gene-of-interest can be effectively activated by crossing reporter plants with plants that express LhG4, allowing the full range of transgene expression to be achieved in the progeny. If this prevents the progeny from being propagated, more experimental material can be generated by crossing the phenotypically normal activator and reporter plants. When GUS was used as the reporter in this system, enzyme activity increased from <0.01 to an average of 10 nmol 4-MU·min⁻¹·mg protein⁻¹ in the presence of pLhG4.

In the pOp/LhG4 system, a gene of interest will be expressed only in cells that express LhG4 and this should depend in turn on the promoter used to express LhG4. So far, only the CaMV 35S-promoter has been tested, but it appears that in this case at least the spatial and quantitative aspects of the promoter can be reproduced by the pOp/LhG4 expression system.

A potential advantage of this expression system is that it may allow a gene of interest to be expressed under a variety of developmental or physiological regimes by simply crossing an initial set of reporter plants with different activator plants that express LhG4 in the appropriate manner. To test this possibility we aim to establish a library of activator plants with diverse patterns of LhG4 expression. Once such activator lines are established, it will overcome the present requirement for a new round of plasmid construction and plant transformation for each expression regime.

As the lac repressor is derived from E. coli it is unlikely that plants contain genes that are regulated by a similar DNA-binding activity. It is also seems unlikely that LhG4 will bind fortuitously near an endogenous plant gene and cause its ectopic expression. The lac repressor recognizes a 20-bp operator sequence and is relatively intolerant of sequence variations (21, 24). Thus the optimal recognition sequence will occur at random only once every $2.8 \times 10^{11}$ bases (assuming equal base frequencies) and a sequence with >10% repressor binding activity is unlikely to occur more than once in $10^{10}$ bases; however it remains to be determined how effectively and

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**Fig. 3.** Reporter expression in the progeny of plants transformed sequentially with reporter construct pOp-GUS and activator construct pLh-G4. Transgenic plants derived from reporter lines 16/12 and 16/56 by retransformation with pLh-G4 were allowed to self. Seeds were germinated in the presence of both hygromycin and methotrexate and GUS activity was assayed in the seedlings. Hygromycin resistant seedlings from each reporter line (16/12 and 16/56) served as controls. (A) A single-stained seedling from the indicated lines is shown. Also shown for comparison are seedlings from an untransformed plant, and a 35S-GUS control plant. (B) Extractable GUS activity was measured in extracts of whole 4-week old axenically grown seedlings from each of the indicated transgenic lines. For each line, four or five samples of six seedlings were assayed.
conditions without external intervention. Secondly high levels of reporter expression can be achieved and sustained reliably, even in tissues that might be inaccessible to inducing chemicals, without the need to continually apply an inducer. Finally spatial control of reporter expression may be achieved by use of appropriate promoters to express LhG4.

Weinmann et al. (10) reported a chimeric plant transcription factor system based on a tet-repressor-VP16 fusion. Reporter expression was efficient in the primary transgens and was sensitive to tetracycline, but it became considerably weaker as the plants matured and these weaker expression levels were sustained in subsequent generations (10). Alternative tRNA-based regulatory systems (26, 27) provide stringent repression of transgene function in the absence of a suppressor tRNA. However, they require specific mutagenesis of the gene-of-interest to introduce a stop codon, achieve only low level synthesis of translation product, and almost certainly interfere with the translation of endogenous mRNAs. Nonetheless, these may be the systems of choice when the transgene product is active at low concentrations and stringent repression is paramount.

In summary, we have developed a system that appears to offer stringent, noninvasive, spatial control of plant gene expression. In future we aim to generate a series of activator lines with LhG4 expressed in various specific tissues. We also envisage that a chemically inducible and spatially controlled expression system may be obtained by bringing LhG4 under steroid hormone control through fusion to the ligand-binding domain of the rat glucocorticoid receptor (25, 28, 29).

**EXPERIMENTAL PROCEDURES**

DNA cloning, manipulation, and analyses were all performed according to standard methods (30). *Agrobacterium tumefaciens* strains were GV3101::pMP90 and GV3101::pMP90RK. Plant material was *Nicotiana tabacum* cv. Petit Havana SR1 (31). Transformation of tobacco was achieved by the leaf-disc method. Hygromycin, kanamycin, and methotrexate were added to culture media at 20 μg/ml, 50 μg/ml, and 0.5 μg/ml, respectively. GUS activity was determined and histochemically localized according to Jefferson (32). Transient expression experiments were performed with tobacco mesophyll protoplasts as described in ref. 33.

**Plasmid Construction.** The coding sequence of the lacI DNA-binding mutant lacI<sup>mut</sup> was obtained from B. Wilcken-Bergmann (University of Cologne), in plasmid pWB100His1 (21). In this clone the lacI<sup>mut</sup> initiation codon had been converted to ATG and incorporated into an Ndel site. The C-terminal coding region of the lacI<sup>mut</sup> mutant was exchanged for that of a lacI derivative that contains a unique SphI site (5'-ACT AGT-3') at codons 329 and 330 of lacI (a gift of S. Oehler and B. von Wilcken-Bergmann [Institut für Genetik, University of Cologne]) to generate plasmid pHisA0. A fragment encoding activation domain II of Ga4 (17) was subcloned from pMA242 (16) and inserted into the unique SpeI site of pHisA0 to generate the in-frame translational fusion, LhG4. To express LhG4 in plants, its coding region was isolated as an Ndel-BamHI fragment and inserted into the Asel and BamHI sites of plasmid pKI102 (I.M., unpublished data) between its CaMV 35S-promoter and polyadenylation signal to generate pKIHisA-Ga4 (pKI102 is derived from pRT101 (34) by replacement of the β-lactamase gene with a kanamycin resistance marker (35) and by filling-in the unique EcoRI site in the polylinker to create an Asel site). The entire expression cassette in pKIHisA-Ga4 was isolated as a PsiI fragment and inserted into the unique PsiI site of binary vector pVK18 to generate pLhG4. Details of pVK18 (I.M., unpublished data) are available on request, but in summary its T-DNA comprises T-DNA right-border followed by the polylinker, bacterial kanamycin resistance marker, and origin of replication of pK19.
(35) and a methotrexate resistance marker for selecting transformed plant cells (36) close to the left-border.

To construct pLH-0, the lacI<sup>po</sup> coding sequence was isolated from pHisA0 (see above) and inserted into pK102 as an NdeI-BglII fragment to generate pKHisA0; a PstI fragment containing the entire expression cassette was isolated from pKHisA0 and inserted into pVK18 to generate pLH-0.

To construct pL-VP16, the lacI-VP16 fusion described by Labow et al. (20) was isolated from plasmid pCMHLP348 as a SacI-BglII fragment and inserted between the CaMV 35S-promoter and polyadenylation signal of expression vector pK102 (see above) cut with SacI and BamHI. A HindIII partial digestion fragment containing the lacI-VP16 fusion plus expression signals was inserted into the HindIII site of pVK18 (see above) to generate pL-VP16.

Construction of Reporter and Control Plasmids pOpGUS and pTA-GUS. These were derived from pVK18 (see above). The methotrexate resistance marker of pVK18 was first replaced by a hygromycin resistance marker (nopaline synthase promoter, hygromycin phosphotransferase gene and a polyadenylation signal) from plasmid pPCV730 (a gift of Csaba Koncz, Max-Planck-Institut, Cologne). A GUS coding sequence with a CaMV polyadenylation signal was isolated from pRTI03GUS as an Xhol-HindIII fragment (37) and inserted between the SacI and HindIII sites in the polylinker adjacent to the right border. Next, a ~100-bp EcoRI-XbaI fragment of pCP6 containing the minimal CaMV 35S-promoter fragment (+8 to −50, see above) with EcoRI, SacI, KpnI, SmaI, and BglII sites upstream was inserted into the EcoRI and XbaI sites upstream of GUS to yield pTA-GUS. Two ideal lac operator sequences (5′-TCTAGAAATTGATCCGTCAACACTAGCAATTGATGAGCGCTCACAATTCTAGA-3′, ref. 24) were obtained from S. Oehler (Institut für Genetik, University of Cologne) in plasmid pWB91010. They were subcloned in the XbaI site of pBluescript-II-SK, reisolated by digestion with EcoRI and ligated into the SacI site to generate pOpGUS. Plasmid pTA-GUS, served as a negative control.

RNA Gel Blot Analysis. Total RNA was isolated from plant tissues based on the procedure of Chomczynski and Sacchi (38), and was dissolved in formamide. Equal concentrations were electrophoresed and blotted according to ref. 39. To detect GUS transcripts, a 1.8kb XbaI-SacI fragment containing the GUS coding region (23) was used. To detect LhG4 and LacI-VP16 transcripts, an Xhol-XbaI fragment was isolated from pKHisA0 (see above). This fragment was chosen as it hybridizes with equal efficiency to LhG4 and LacI-VP16.