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TECHNICAL ADVANCE

A glucocorticoid-mediated transcriptional induction system in transgenic plants

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Summary

A novel chemical induction system for transcription in plants has been developed, taking advantage of the regulatory mechanism of vertebrate steroid hormone receptors. A chimeric transcription factor, designated GVG was constructed, consisting of the DNA-binding domain of the yeast transcription factor GAL4, the transactivating domain of the herpes viral protein VP16, and the receptor domain of the rat glucocorticoid receptor (GR). The *GVG* gene was introduced into transgenic tobacco and *Arabidopsis* together with a luciferase (*Luc*) gene which was transcribed from a promoter containing six tandem copies of the GAL4 upstream activating sequence. Induction of luciferase activity was observed when the transgenic tobacco plants were grown on an agar medium containing dexamethasone (DEX), a strong synthetic glucocorticoid. Induction levels of the luciferase activity were well correlated with DEX concentrations in the range from 0.1 to 10 μM and the maximum expression level was over 100 times that of the basal level. Analysis of the induction kinetics by Northern blot analysis showed that the *Luc* mRNA was first detected 1 h after DEX treatment and increased to the maximum level in 4 h. The stationary induction level and the duration of the induction varied with the glucocorticoid derivative used. The *GVG* gene activity can also be regulated by DEX in transgenic *Arabidopsis* plants. The results indicate that a stringent chemical control of transcription can be achieved in plants with the GVG system. Advantages and potential uses of this system are also discussed.

Introduction

Transgenic techniques have become a powerful tool to address important biological problems in multicellular organisms, and this is particularly true in the plant field, as transgenic plants are easier to produce than transgenic animals. Many approaches that were impossible to implement by traditional genetics can now be realized by transgenic techniques, including the introduction of homologous or heterologous genes into plants, with modified functions and altered expression patterns. In most experiments, the transgenes are transcribed from a strong promoter, such as the 35S promoter of the cauliflower mosaic virus (CaMV). However, a more flexible gene expression system is needed to extract greater benefits from transgenic technology. Good inducible transcription systems are desired because transgenic plants with inducible phenotypes are as useful as conditional mutants isolated by traditional genetics. In this regard, several induction systems have been reported and successfully used (Ainley and Key, 1990; Gatz *et al.*, 1992; Mett, *et al.*, 1993; Weinmann *et al.*, 1994). Among these, the tetracycline-dependent expression systems are the most advanced (for review, see Gatz, 1996).

The glucocorticoid receptor (GR) is a member of the family of vertebrate steroid hormone receptors. GR is not only a receptor molecule but also a transcription factor which, in the presence of a glucocorticoid, activates transcription from promoters containing glucocorticoid response elements (GREs) (for reviews, see Beato, 1989; Picard, 1993). It has been considered that the GR system could be a good induction system in plants because it is simple, and glucocorticoid itself does not cause any pleiotropic effects in plants. Nevertheless, a general and efficient glucocorticoid-inducible system using GR has not yet been constructed for transgenic plants, although it has been demonstrated that a system comprising GR and GREs could work in a transient expression system with cultured plant cells (Schena *et al.*, 1991). On the other hand, it has been reported that the hormone-binding domain (HBD) of GR could regulate the function of plant transcription factors in transgenic plants (Aoyama *et al.*, 1995; Lloyd *et al.*, 1994).

In this paper, we describe the construction of a transcriptional induction system using not the entire GR protein but only its HBD as a regulatory domain in a chimeric transcription factor. The transcription factor, designated GVG, also contains heterologous DNA-binding and transactivating domains from the yeast transcription factor GAL4 and the herpes viral protein VP16, respectively. The *GVG* gene was introduced into tobacco together with a luciferase (*Luc*) reporter gene transcribed from a promoter containing

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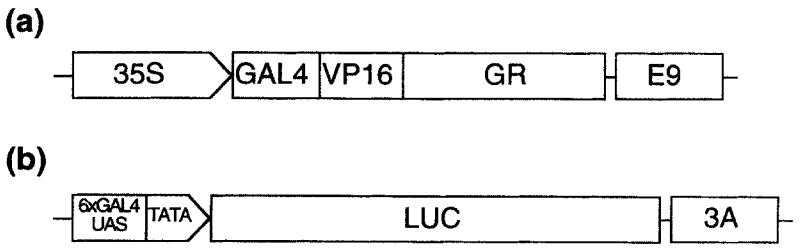


Figure 1. Structures of the *trans*-acting factor and *cis*-acting element in the GVG system. (a) Structure of the *GVG* gene. The DNA fragments encoding the chimeric transcription factor GVG were placed between the cauliflower mosaic virus 35S promoter (Odell *et al.*, 1985) and the poly(A) addition sequence of the pea ribulose bisphosphate carboxylase small subunit rbcS-E9 (Coruzzi *et al.*, 1984). (b) Structure of the *Luc* reporter gene. The luciferase-coding fragment is preceded by a promoter containing six copies of the GAL4 UAS and the −46 to +1 region of the 35S promoter and flanked at the 3′ end by the poly(A) addition sequence of the pea rbcS-3A (Fluhr *et al.*, 1986).

six copies of the GAL4 upstream activating sequence (GAL4 UAS). Good induction of both the luciferase activity and the *Luc* mRNA levels were observed upon glucocorticoid treatment. The advantages and potential uses of the glucocorticoid-inducible system are discussed.

Results and discussion

Construction of trans- *and* cis-*acting factors of the GVG system*

Hormone-binding domains (HBDs) of vertebrate steroid hormone receptors are thought to have repressive effects on covalently linked, neighboring domains in the absence of their cognate ligands, and binding of the appropriate ligand to a HBD results in de-repression (Picard, 1993). We took advantage of this mechanism to design a transcription factor in which a constitutively active transactivating function was regulated by the HBD of the rat GR in *cis* (Picard *et al.*, 1988; Rusconi and Yamamoto, 1987). As a constitutively active transactivating function, we chose a chimeric transcription factor comprising the DNA-binding domain of the yeast transcription factor GAL4 (Keegan *et al.*, 1986) and the transactivating domain of the herpes viral protein VP16 (Triezenberg *et al.*, 1988). This chimeric protein, GAL4–VP16, was thought to act as a strong transcription factor in all cell types because the activation domain of VP16 is known to interact directly with general transcription factors, which are thought to be evolutionarily conserved among eukaryotes (Goodrich *et al.*, 1993; Lin *et al.*, 1991; Sadowski *et al.*, 1988). Indeed, our preliminary experiments using transient expression assays with particle bombardment showed that GAL4–VP16 acted as a strong transcription activator in plants (data not shown). It has been shown that the HBD of the human estrogen receptor could regulate similar chimeric transcription factors in yeast and animal tissue culture cells (Braselmann *et al.*, 1993; Louvion *et al.*, 1993). We therefore added the HBD of the rat GR to this chimeric transcription factor and the resulting hybrid transcription factor was designated 'GVG' because it consisted of one domain each from GAL4, VP16 and GR. A DNA fragment encoding the GVG transcription factor was placed between the cauliflower mosaic virus 35S promoter (Odell *et al.*, 1985) and the poly(A) addition sequence of the pea ribulose bisphosphate carboxylase small subunit rbcS-E9 (Coruzzi *et al.*, 1984) (Figure 1a).

As a binding site for GVG, a DNA fragment containing six copies of the GAL4 UAS (Giniger *et al.*, 1985) was fused 5′ to the minimal −46 to +9 region of the CaMV 35S promoter (Figure 1b). A DNA fragment encoding the firefly luciferase (*Luc*) gene (de Wet *et al.*, 1987) flanked at the 3′ end by the poly(A)-addition sequence of the pea rbcS-3A (Fluhr *et al.*, 1986) was placed downstream of the promoter to monitor the transcriptional induction (Figure 1b). Both the GVG and the luciferase constructs were inserted into a plasmid vector derived from pMON721 (Monsanto Co.) and introduced into tobacco plants using *Agrobacterium*-mediated transformation. Fourteen independent primary transgenic tobacco plants carrying both the *GVG* gene and the *Luc* gene were obtained. Of these, plants from 11 lines showed significant induction of luciferase activity by dexamethasone (DEX) treatment (data not shown). Induction experiments were carried out with homozygous T_3 plants, and results with plants from a single transgenic line, which exhibited a typical response to glucocorticoid, are described below.

Induction of luciferase activity in transgenic plants

We first measured stationary induction levels of the luciferase activity in response to different concentrations of a glucocorticoid. Young transgenic plants grown on an agar medium were transferred to a fresh agar medium containing different concentrations of DEX, a strong, synthetic glucocorticoid. After 2 days on the induction medium, whole cell lysate was prepared from 10 plants and assayed for luciferase activity. Figure 2 shows an image of luciferase luminescence from plants using a high-sensitivity camera system (a) and the relative luciferase activity induced by different concentrations of DEX (b). The luciferase activity

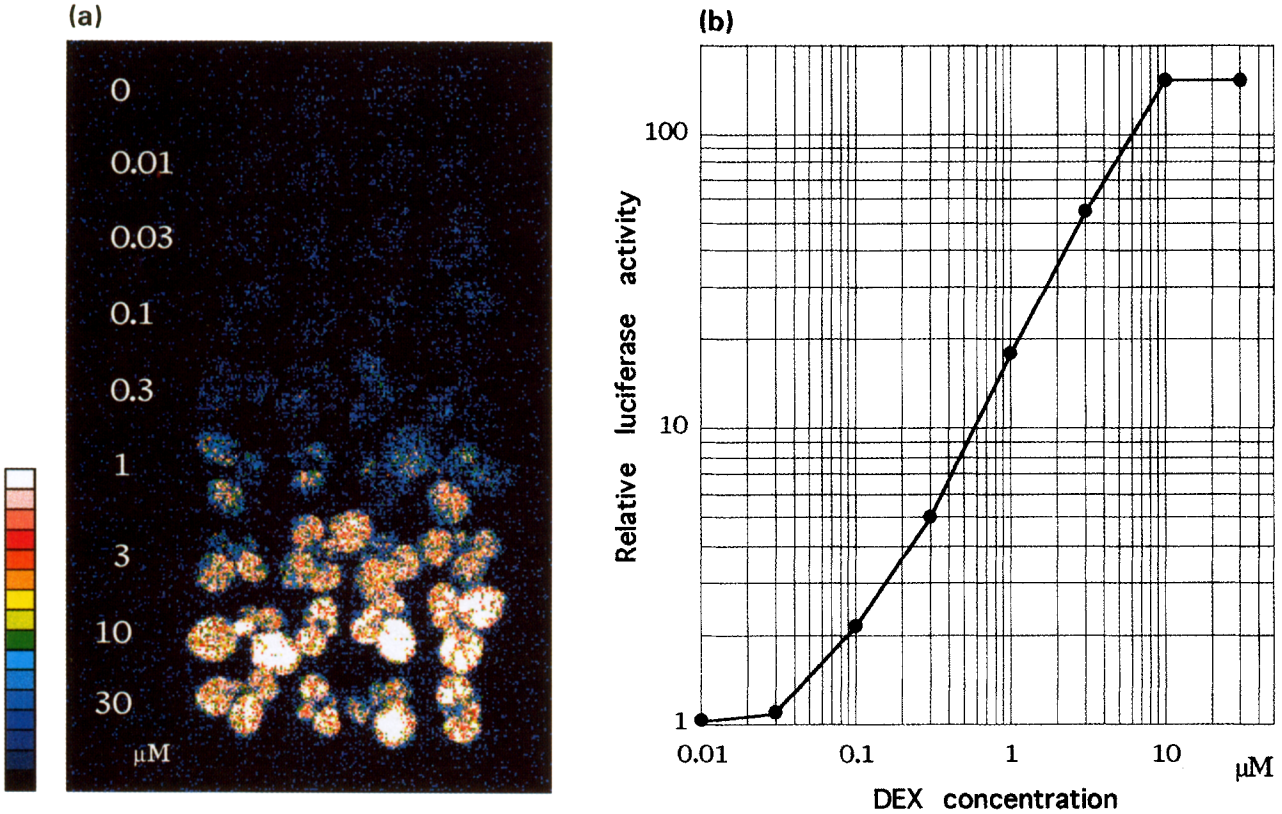


Figure 2. Stationary expression levels of the luciferase activity induced by different concentrations of DEX.
Transgenic tobacco plants carrying the *GVG* gene and the *Luc* reporter gene were first grown on agar medium for 14 days and then transferred to a fresh agar medium containing different concentrations of DEX for an additional 2 days.
(a) The luciferase luminescence from plants was imaged using a high-sensitivity camera system (Hamamatsu Photonic Systems). The color scale on the left shows the luminescence intensity of from dark blue (lowest) to white (highest).
(b) Relative luciferase activities were plotted against DEX concentrations. The value obtained at 0 μM DEX (the basal, non-induction level) was arbitrarily set as 1.

detected in the absence of DEX was very low and comparable to that obtained from transgenic plants carrying a luciferase gene preceded by the TATA region only (data not shown). This result indicates that the GAL4 UAS was quiescent in plants and not recognized by any endogenous plant transcription factor. Induction was detectable at a concentration of 0.1 μM DEX or higher, and a good correlation between DEX concentrations and induction levels was obtained in the concentration range from 0.1 to 10 μM. The maximum induction level was 100 times the basal level.

In this experiment, plants were treated with DEX for a sufficiently long period to ensure that the luciferase activity had reached a plateau for each DEX concentration. Induction was very slow in plastic wares, as observed in this experiment, probably because, under the enclosed conditions, transpirational water flow in plants and hence the uptake of glucocorticoid through the roots was slow compared with that under non-enclosed, open-air conditions. On the other hand, under the latter conditions, it is very difficult to precisely control the glucocorticoid concentration in plants because the hormone rapidly accumulates in leaves, as a result of transpiration.

Kinetics of the transcriptional induction by DEX

Although the luciferase activity is easy to measure, it is not suitable for kinetic study within a short time scale because the half-life of luciferase activity is estimated to be approximately 3 h (Thompson *et al.*, 1991). To obtain more direct information on the kinetics of induction, total RNA was prepared and subjected to Northern blot analysis. In these experiments, plants were placed in the open air to ensure rapid DEX uptake. Transgenic plants were adapted to hydroponic growth conditions in the open air and DEX was added to the liquid growth medium at a final concentration of 10 μM. Total RNA was prepared from 20 plants at each time point and subjected to Northern blot analysis. Figure 3 shows that the *Luc* mRNA was first detected 1 h after the addition of DEX and the amount increased to a stationary level within the next 3 h. To examine the sustainability of the induction, DEX was removed from the medium and total RNA prepared from the plants was analyzed. Figure 3 shows that *Luc* mRNA could be detected even 4 days after removal of DEX.

A similar result was obtained by monitoring the luciferase

activity. Due to the high sensitivity of detection, the induced luciferase activity could be measured 30 min after DEX addition and for 8 days after removal of the hormone (data not shown). From these results, we can conclude that the transcriptional induction by DEX is rapid and can be maintained for a long period.

Responses to various glucocorticoids

Different glucocorticoid derivatives were examined for the intensity and the duration of induction. Young transgenic plants grown on an agar medium were transferred to a fresh agar medium containing 30 μM of different glucocorticoids and grown for an additional 2 days. After the induction, plants were returned to the agar medium without glucocorticoid. At each time point indicated in Figure 4, 10 plants were harvested and their luciferase activities assayed. Figure 4 shows that the induction levels and their durations were different with different glucocorticoid derivatives. The highest induction levels was obtained with either DEX or triamcinolone acetonide. In contrast, only low or moderate induction levels were detected with betamethasone or hydrocorticoid, respectively. In these experiments, we assumed that the induction level obtained with each glucocorticoid had reached a steady-state level because longer induction periods did not significantly increase the luciferase activity (data not shown). The induction by DEX was maintained for a longer period compared with that by triamcinolone acetonide, whereas both glucocorticoids conferred about the same induction level at the beginning of the treatment. Although the stability of these glucocorticoids in plants is not known in these experiments, the induction characteristics of different glucocorticoids might be used to regulate the intensity and the duration of induction.

Advantages and potential uses of the GVG system

So far, the regulatory mechanism of GR has been utilized in inducible transcription systems of animal and yeast cells

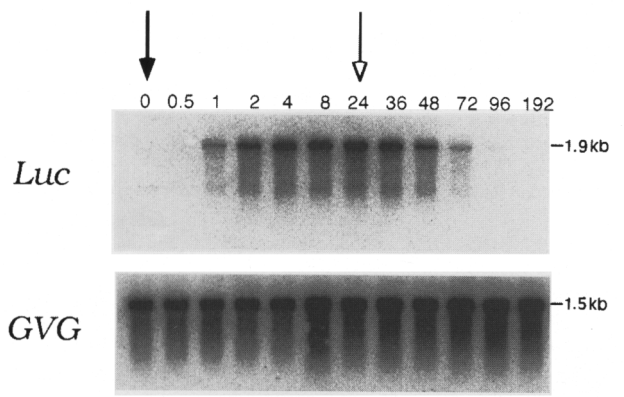


Figure 3. Kinetics of the *Luc* mRNA level induced by DEX.
Transgenic tobacco plants carrying the *GVG* gene and the *Luc* reporter gene were first grown on agar medium for 14 days and then adapted to growth in a hydroponic medium for 3 days. DEX treatment was started by adding DEX to the medium at a final concentration of 10 μM (time indicated as 0). After 24 h of treatment, DEX was then removed from the medium. Total RNA was prepared from 20 plants at each time indicated and subjected to Northern blot analysis. cDNA fragments of the firefly luciferase and the *GVG* gene were used as probes. Signals were imaged by the BAS-2000 system (Fuji Photo Films Co.). Closed and open arrows indicate the time points of adding and removing DEX, respectively.

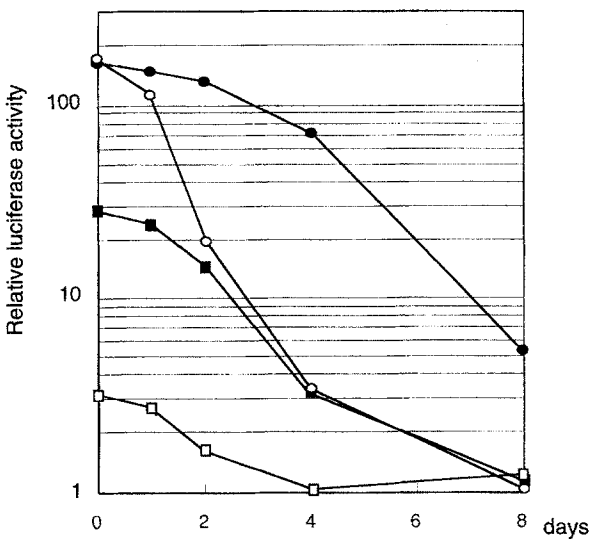


Figure 4. Intensity and sustainability of induction by various glucocorticoids.
Transgenic tobacco plants carrying the GVG gene and the *Luc* reporter gene were first grown on agar medium for 14 days and then transferred to a fresh agar medium containing 30 μM of different glucocorticoids for an additional 2 days. After the induction, plants were transferred back to the agar medium without glucocorticoid (time indicated as 0). Relative luciferase activities induced by DEX (●), triamcinolone acetonide (○), betamethasone (■) and hydrocortisone (□) were plotted. The value obtained with no glucocorticoid (the non-induction level) was arbitrarily set as 1.

Figure 6. Induction of luciferase activity in *Arabidopsis.*
The induction experiment was done with transgenic *Arabidopsis* carrying the *GVG* and *Luc* genes.
(a) A transgenic plant grown in a pot for 3 weeks was sprayed with a solution containing 0.5 mM potassium luciferin and 0.01% (w/v) Tween-20 and assayed for luciferase activity.
(b) The same plant was then sprayed with a solution containing 30 μM DEX and 0.01% (w/v) Tween-20. Twenty-four hours later, the plant was sprayed again with the luciferin solution and assayed.
In both cases, the luminescence from the plant was imaged using a high-sensitivity camera system (Hamamatsu Photonic Systems). Heterogeneity of the luminescence seen in the plant treated with DEX was caused by uneven absorption of luciferin. The color scale on the left shows the luminescence intensity from dark blue (lowest) to white (highest).

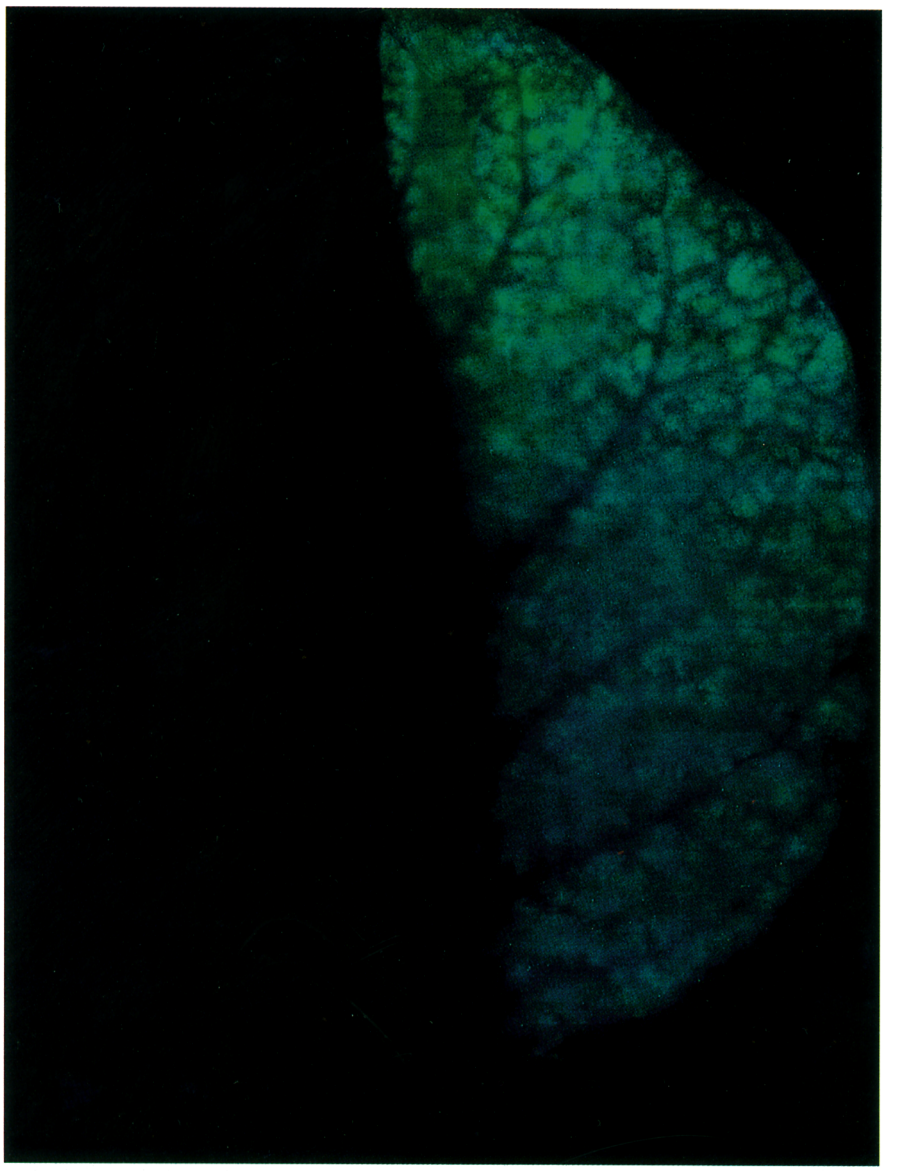
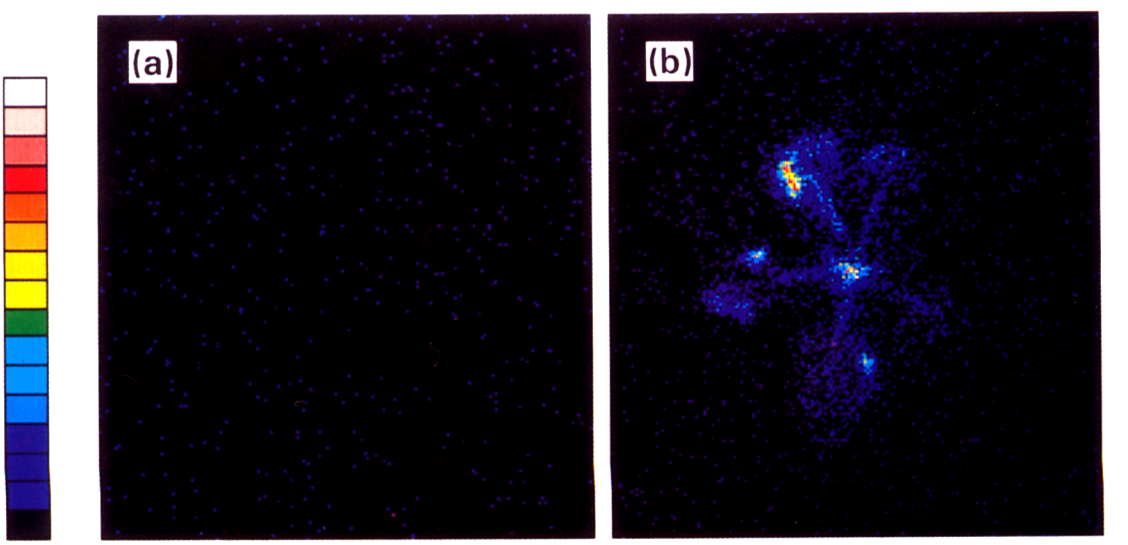


Figure 5. Local induction of luciferase expression by glucocorticoid spraying.
The right and left halves of a leaf (about 10 cm in length) on a mature plant carrying the *GVG* and the *Luc* genes were sprayed with a solution containing 30 μM DEX and 0.01% (w/v) Tween-20 and a control solution, respectively. Twenty-four hours after spraying, the leaf was excised and allowed to take up luciferin through the petiole. The picture was taken by placing an instant color film (Fuji Photo Films Co. LP100) on to the leaf, with thin plastic film in between them, for 5 h.



(Picard, 1993); here, we demonstrated that the mechanism can also operate in transgenic plants. A major advantage of the GVG system in plants is the fact that GR and glucocorticoid, at least at the concentrations used, are non-toxic and have no observable adverse physiological effects on plants, thus allowing the induction of target genes without pleiotropic effects. To retain this advantage, all the other components in the GVG system were also obtained from non-plant sources.

A further advantage of the system is that glucocorticoid possesses characteristics that make it suitable as an inducer chemical. Because glucocorticoid can easily permeate plant cells, rapid gene induction can be performed using various methods. As shown in Figure 5, a local induction of gene expression can be obtained simply by spraying with a glucocorticoid solution. It is clear that inducer chemicals accumulate in leaves to a high concentration when whole plants are treated under open air conditions. Even under such conditions, the accumulated glucocorticoid does not cause any visible damage to leaves (data not shown). As shown here, the induction level can be regulated by using different concentrations or different derivatives of glucocorticoid. This feature may be very helpful for analyzing dose-dependent effects of induced gene products. Glucocorticoid is one of the best-studied biological compounds and over 100 different types of glucocorticoid derivatives are now available from commercial sources. Some of the glucocorticoid derivatives may be very stable in plants whereas others are rapidly degraded. These types of glucocorticoid would be useful for stable and transient induction, respectively. Moreover, some glucocorticoid antagonists might be used for down-regulation of induction.

The GVG system developed here is very flexible in its composition. For example, the transcriptional induction can be limited to a specific tissue by replacing the 35S promoter for the *GVG* gene with a tissue-specific promoter. Each functional domain in the GVG fusion protein is also exchangeable, allowing further refinement of the system. With a different DNA-binding domain and the HBD of another steroid hormone receptor, it is possible to develop another steroid induction system that can be used in combination with the GVG system.

Various plant species have been employed for studies on basic and applied aspects of plant sciences, and among them, *Arabidopsis* has emerged as a model plant for basic explorations of plant biology. So far, however, good induction systems have not yet been developed for this model plant. Induction systems using plant promoters, e.g. heat-shock promoters, are not suitable because they elicit pleiotropic effects. Although the tetracycline-dependent expression system has been successfully used in tobacco, it does not appear to function in *Arabidopsis* (Gatz, 1996). On the other hand, we found that our GVG system can also function in *Arabidopsis*. Figure 6 shows that the luciferase activity in transgenic *Arabidopsis* was induced effectively by DEX.

In this report, we have described the construction of a novel glucocorticoid-inducible transcriptional system and investigated in some detail its induction characteristics in transgenic tobacco. The transcription of genes other than *Luc* can also be induced effectively by the GVG system in transgenic tobacco as well as in *Arabidopsis* (data not shown). We believe that the GVG system is widely applicable to many genes and in different species of transgenic plants.

Experimental procedures

DNA constructs

Plasmids derived from pMON721 (Monsanto Corporation, St Louis, MO) and pBI101 (Clontech Laboratories, Inc.) were used as vectors for work with tobacco and *Arabidopsis*, respectively. The *GVG* gene, which was transcribed from the −343 to +1 region of the CaMV 35S promoter (Odell *et al.*, 1985), was flanked at the 3′ end by the poly(A) addition sequence of the pea ribulose bisphosphate carboxylase small subunit rbcS-E9 (Coruzzi *et al.*, 1984). The DNA fragments encoding specific domains were produced by the polymerase chain reaction (PCR) using primers of appropriate sequences for in-frame cloning. The GAL4 DNA binding domain comprises amino acids 1–74 (Laughon and Gesteland, 1984), the VP16 acidic domain comprises amino acids 413–490 (Dalrymple *et al.*, 1985), and the GR receptor domain comprises amino acids 519–795 (Miesfeld *et al.*, 1985). The GAL4 UAS DNA (5′-CGGGTGACAGCCCTCCG-3′) was synthesized chemically and the coding sequence for the *Luc* gene (de Wet *et al.*, 1987) was excised from pGEM-luc (Promega Co.). The *Luc* coding sequence was transcribed from six copies of GAL4 UAS placed 5′ to the −46 to +1 region of the 35S promoter and flanked at the 3′ end by the poly(A) addition sequence of the pea rbcS-3A (Fluhr *et al.*, 1986).

Transgenic plants

pMON721 and pBI101 derivatives were introduced into *Agrobacterium tumefaciens* strain ABI (Monsanto Corporation, St Louis, MO) and strain LBA4404 (Clontech Laboratories, Inc.), respectively. Leaf discs of *Nicotiana tabacum* cv SR1 were transformed and regenerated as described by Horsch *et al.* (1988) and transformation of *Arabidopsis* was performed according to the method of Valvekens *et al.* (1988). Primary transgenic plants were allowed to self-fertilize and seeds were collected. The transgenic progeny were germinated on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.8% agar and 100 μg ml^{-1} kanamycin for selection. Unless otherwise noted, T_3 homozygous plants grown on the same agar medium for 14 days after germination were used in induction experiments. In some experiments, plants were transferred to a hydroponic growth medium containing 1/100 concentration of MS salts and adapted to the growth conditions for 3 days before used. In all cases, plants were exposed to continuous light and a temperature of 27°C (tobacco) or 22°C (*Arabidopsis*).

Glucocorticoid treatments

All glucocorticoid derivatives, dexamethasone (DEX), triamcinolone acetonide, betamethasone and hydrocortisone were pur-

chased from Wako Pure Chemical Industries. The chemicals were dissolved in ethanol at 30 mM before use and diluted in either the growth medium or the spraying solution. The same volume of ethanol was added to negative control medium or solution. In the case of whole-plant treatment, plants were grown on an agar medium containing glucocorticoid or their roots were submerged in a hydroponic growth medium containing glucocorticoid. For the spraying method, the solution contained 30 μM DEX and 0.01% (w/v) Tween-20; the latter was added as a wetting agent. In experiments involving spraying of one half of a leaf, the other half and other parts of the plant were covered with a plastic film.

Luciferase assays

Extraction of luciferase and assays for relative luciferase activities were carried out as described by Millar *et al.* (1992). To image the luciferase luminescence, roots of plants treated with DEX were submerged in a solution containing 0.5 mM potassium luciferin (Sigma) for 1 h or the petiole of a sprayed leaf was submerged in a solution of 0.5 mM potassium luciferin for 30 min. Potted plants were sprayed with a solution containing 0.5 mM potassium luciferin and 0.01% (w/v) Tween-20 and left for 30 min. The luciferase luminescence from plants was visualized using an image-intensifying camera (VIM) and photon-counting image processors (ARGUS-50) purchased from Hamamatsu Photonic Systems. The exposure time was 10 min. To take a picture of the luciferase luminescence from the sprayed leaf, the leaf and an instant color film (LP100, Fuji Photo Films Co.) were placed in contact with one another, with a thin plastic film between them, for 5 h.

RNA analysis

Total RNA isolation and Northern blot hybridization were performed as described by Nagy *et al.* (1988). After hybridization, signals were imaged with the BAS-2000 system (Fuji Photo Films Co.).

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