

A glucocorticoid-inducible gene expression system can cause growth defects in tobacco

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Abstract We find that an expression system widely used to chemically induce transgenes of interest in tobacco (*Nicotiana tabacum* Petit Havana SR1) can cause severe growth defects in this species. This gene expression system has been shown to cause non-specific effects (including growth retardation) in other plant species, but has until now been largely accepted to be a relatively problem-free system for use in tobacco. The expression system is based on the ability of the glucocorticoid dexamethasone (DEX) to activate a non-plant chimeric transcription factor (GVG), which then activates expression of a transgene of interest. The aberrant growth phenotype only manifests itself after DEX application and only occurs in plants in which the constitutive levels of GVG expression are higher than average. We found that ~30% of all transgenic plants produced showed some level of growth retardation under our standard growth conditions. However, by modulating irradiance levels following DEX application, we also showed that the manifestation and severity of the aberrant phenotype is highly dependent upon growth conditions, highlighting that such conditions are a critical parameter to consider during all stages of using this gene expression system. We also identified an increase in ACC oxidase gene expression as an early, sensitive and robust molecular marker for the aberrant phenotype. This molecular marker should be valuable to investigators wishing to

readily identify transgenic plants in which GVG expression levels are beyond a threshold that begins to produce non-specific effects of the gene expression system under a defined set of growth conditions.

Keywords Aberrant phenotype · ACC oxidase · Dexamethasone · GVG transcription factor · Irradiance · *Nicotiana tabacum*

Abbreviations

ACC 1-Aminocyclopropane-1-carboxylic acid
AOX Alternative oxidase
DEX Dexamethasone
FeSOD Iron superoxide dismutase

Introduction

Systems to chemically regulate the expression of transgenes represent powerful tools for both basic and applied plant biology research (Zuo and Chua 2000; Padidam 2003; Moore et al. 2006). In general, such systems have two components: a transcription factor, the activity of which is responsive to a chemical; and a response element, through which the transcription factor controls the expression of a gene of interest.

One of the chemical-regulated gene expression systems most utilized in plants to date was described by Aoyama and Chua (1997), and we refer to it here as the GVG expression system. The first component of this system is a chimeric transcription factor, coined GVG, that contains the DNA binding domain of the yeast GAL4 transcription factor, a herpes viral VP16 transactivation domain and the receptor domain of the rat glucocorticoid receptor. GVG is constitutively

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expressed in the plant by the CaMV 35S promoter, but is only active following application of the glucocorticoid dexamethasone (DEX), which binds to the receptor domain, allowing movement of activated GVG to the nucleus. The second component of the system consists of one's transgene of interest, the expression of which is controlled by a promoter containing six GAL4 DNA binding sites (Aoyama and Chua 1997).

An important prerequisite to studying how the induced expression of a gene of interest impacts a biological system is to be sure that the components of the inducible expression system itself do not significantly impact the biology of the organism. In the case of the inducible expression system described above, the transcription factor (GVG) and the chemical inducer (DEX) are both foreign to plants and therefore represent potentially excellent components to utilize for such a system. Nonetheless, the GVG expression system has been shown to cause severe side effects in several plant species (such as *Arabidopsis thaliana*; Kang et al. 1999) and we report here that this is similarly the case in *Nicotiana tabacum*.

Materials and methods

Plasmid constructs

Standard recombinant DNA techniques were performed according to Sambrook et al. (1989). The cDNA clone pAONT1 (*EcoRI* fragment) contains the complete coding region of tobacco *Aox1* (1059 bp) along with an additional 64 nucleotides at the 5' end and 273 nucleotides at the 3' end (Vanlerberghe and McIntosh 1994). This cDNA (encoding mitochondrial alternative oxidase [AOX]) was subcloned into the M13 vector pUC119 and used for mutagenesis. Oligonucleotide-directed in vitro mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis kit, according to the manufacturer's instructions (Stratagene, California). The complementary oligonucleotides 5'-GAATGGAAATGGAATGAATTTAGGCCTTGGGAGACGTAC-3' and 5'-GTACGTCTCCCAAGGCCTAAATTCATTCCATTTCATTTC-3' were used to change Cys₁₂₆ to Glu and to introduce the unique restriction site *StuI* (silent mutation). Clones verified by restriction digest analysis to contain the *StuI* restriction site were then subjected to DNA sequence analysis to confirm that the correct mutations were indeed present and that no other unexpected mutations had occurred. Primers (forward 5'-GCTCGAGCCAAGTTTCTTTCC-3'; reverse 5'-CTCATTGTGCACTAGTGCTATCTCAG-3') were then

used to add an *XhoI* restriction site to the 5' end and a *SpeI* restriction site to the 3' end of both the mutated and native *Aox1* clones. These were then directionally cloned into the binary plasmid pTA7001, which contains the complete two-component glucocorticoid-inducible gene expression system (Aoyama and Chua 1997). The final constructs used (including an empty vector control) are outlined in Fig. 1a.

Transgenic plants

The binary vectors described above were then introduced into *Agrobacterium tumefaciens* LBA4404 and used to transform tobacco (*Nicotiana tabacum* cv. Petit

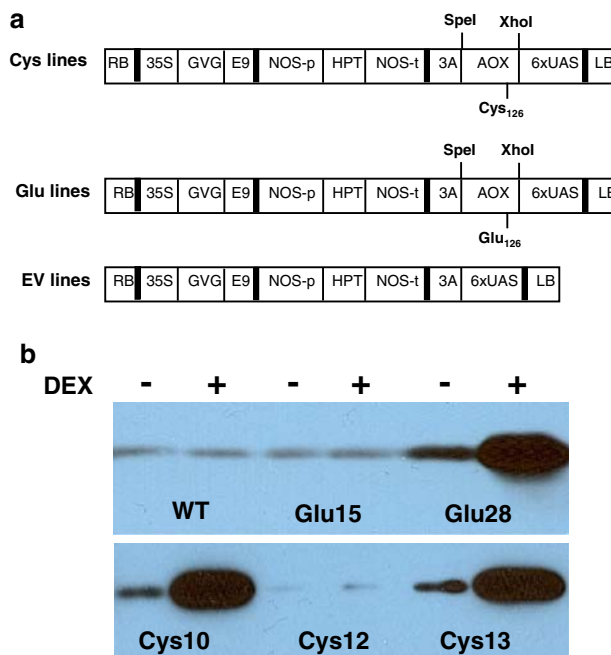


Fig. 1 **a** The three constructs used for plant transformation. *Cys* lines will inducibly express the native AOX, which includes a Cys residue at position 126. *Glu* lines will inducibly express a recombinant AOX in which Cys₁₂₆ is replaced by Glu. *EV* lines are empty vector control lines, which contain all components of the GVG expression system, but not an AOX transgene. See text for further details. *RB* right T-DNA border; *35S* CaMV 35S promoter; *GVG* chimeric transcription factor; *E9* pea ribulose biphosphate carboxylase small subunit E9 transcription termination sequence; *NOS-p* nopaline synthase promoter; *HPT* hygromycin phosphotransferase; *NOS-t* nopaline synthase transcription termination sequence; *3A* pea ribulose biphosphate carboxylase small subunit 3A transcription termination sequence; *AOX* alternative oxidase cDNA; *6xUAS* six copies of GAL4 UAS fused 5' to the terminal -46 to +9 region of the CaMV35S promoter; *LB* left T-DNA border. **b** AOX protein levels in WT and select transgenic lines. Mitochondria were isolated from 8–10-week-old plants that had been sprayed for 24 h in the presence or absence of 30 μM DEX. Mitochondrial proteins were then separated by SDS-PAGE, transferred to nitrocellulose and probed with a monoclonal antibody against AOX. See text for further details

Havana SR1) by a leaf disc method (Horsch et al. 1986).

With the Cys, Glu and EV constructs (Fig. 1a), 34, 60 and 17 primary transformants were generated, respectively. Analysis of segregation ratio for resistance to hygromycin revealed that the progeny from 7 (out of 34), 17 (out of 60) and 7 (out of 17) of these lines contained T-DNA insertion at a single locus. Homozygous progeny from the second generation of these lines were used for all experiments.

Growth conditions

Plants were raised in controlled-environment growth chambers (Model PGR-15, Conviron, Winnipeg, Canada) with a 16 h photoperiod, a temperature of 28°C/22°C (light/dark) and a relative humidity of 60%. The plants were raised at an irradiance of approximately 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For some experiments, the plants were also transferred to a lower irradiance (approximately 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) following DEX application (see text for details). The plants were grown in a general purpose growing medium (Pro-mix BX, Premier Horticulture Ltd., Rivière-du-Loup, Quebec, Canada) and were irrigated with water or a 10× diluted Hoagland's solution as necessary.

Glucocorticoid treatments

DEX was sprayed on plants as described by Aoyama and Chua (1997). Briefly, a 30 mM stock solution of DEX (Sigma) in ethanol was diluted in water to a final concentration of 30 μM , adding 0.01% (w/v) Tween–20 as a wetting agent. The control plants were sprayed with the same solution minus DEX. The plants were taken from the growth chambers and all leaves were sprayed thoroughly until the upper and lower surfaces were soaked. The plants were then allowed to dry for 1 h before transfer back to the growth chamber.

For some experiments, the seeds were germinated and grown on plates containing an agar-solidified medium in the presence or absence of DEX. The agar medium (pH 5.7) contained Murashige and Skoog salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 3% (w/v) sucrose and 0.7% (w/v) phytagar. After autoclaving, DEX (30 μM final concentration) was added to the cooled media just prior to pouring the plates. The seeds were first surface-sterilized in 70% ethanol for 1 min and then in 10× diluted bleach for 10 min, followed by four washes with sterile distilled water. After placing the seeds (30–40 seeds/plate), the plates were kept at room temperature under continuous low fluorescence light ($\sim 40 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 23

days. The young seedlings were then carefully removed from the agar and their root length measured using calipers.

Mitochondrial isolation and analysis

The isolation of Percoll-gradient purified tobacco leaf mitochondria and immuno-blot analysis of AOX protein levels were done as previously described (Vanlerberghe et al. 1995; Robson and Vanlerberghe 2002).

Northern blot analyses

To generate hybridization probes for Northern analyses, partial cDNAs for GVG, iron superoxide dismutase (Fe-SOD) and 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase were amplified from tobacco leaf RNA. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. In the case of GVG, the source RNA was from a transgenic plant expressing GVG. Partial cDNAs were amplified using a reverse transcription-PCR kit (Access RT-PCR, Promega) and cloned into pGEM-T Easy (Promega, Madison, WI). Primers for the RT-PCR were designed based upon sequence data for GVG (AF294979), a tobacco Fe-SOD (M55909; Van Camp et al. 1990) and available ACC oxidase sequence for different tobacco cultivars (AY426756; AY905606; X98493; Z29529). For GVG, the primers used were forward 5'-CGCTACTCTCCCAAACC-3' and reverse 5'-TCATATCCTGCATACAACACC-3'. For FeSOD, the primers were forward 5'-CTCCAGCCTCCTCC TTATCC-3' and reverse 5'-TCGTGCCTGCTAGAT TTGC-3'. For ACC oxidase, the primers (including one degenerate primer) were forward 5'-TCTTGAAG GC/TGTAC/GAAGC-3' and reverse 5'-TTAACGAC GATGGAGTGG-3'.

After confirmation of their identity by sequencing, the above cDNAs were excised from pGEM-T Easy, purified from agarose gels using a gel extraction kit (Qiagen), radiolabeled using the Rediprime II random priming labeling system (Amersham) and used as hybridization probes for Northern analyses. Total leaf RNA (20 μg , isolated as above) was separated on formaldehyde agarose gels and transferred to Hybond N membrane (Amersham Pharmacia). Only RNA judged to be of high quality and equally loaded between lanes was used. This was judged by O.D. $^{260/280}$ ratios and by visualization of the ethidium bromide stained gel prior to transfer. Pre-hybridization and hybridization were each done overnight at 65 °C in 0.25M Na_2HPO_4 (pH 7.2) with 7% w/v SDS. Pre-hybridization also included denatured salmon sperm

DNA (100 µg ml⁻¹). Washes were performed according to Church and Gilbert (1984). Blots were analyzed by autoradiography using CL-Xposure film (Pierce) and a Biomax Transcreen-HE intensifying screen (Kodak). After x-ray film development, Northern blots were quantified by densitometry using an imaging system (Alpha Innotech Corporation) and associated software (AlphaEaseFc).

Results

AOX is a mitochondrial inner membrane-localized protein that functions as a non-energy conserving terminal oxidase in the respiratory electron transport chain of plants and other organisms (Finnegan et al. 2004; McDonald and Vanlerberghe 2006). As part of our efforts to understand biochemical mechanisms that control AOX activity, we sought to inducibly express different recombinant AOX proteins *in planta* using the GVG expression system originally described by Aoyama and Chua (1997). These recombinant proteins included one in which a Cys residue (Cys₁₂₆) believed to have an important regulatory role in controlling AOX activity (Rhoads et al. 1998; Vanlerberghe et al. 1998) was changed to Glu. In general, we wished to examine how the induced expression of this protein with altered regulatory properties would impact plant growth and development. However, during the course of that work, it became evident that the expression system itself was dramatically impacting a significant percentage of the plants. This phenomenon was investigated further and is the subject of this manuscript.

Transgenic tobacco plants containing the GVG expression system

Figure 1a outlines the three gene constructs used to generate transgenic plants. One construct was used for DEX-inducible expression of a recombinant AOX in which Cys₁₂₆ had been changed to Glu (referred to as Glu lines), one construct was used for DEX-inducible expression of the native AOX (referred to as Cys lines) and the third construct was an empty vector control (referred to as EV lines) that includes all the components of the GVG expression system, but lacks the transgene of interest. We included a total of 29 independent transgenic lines in our analysis (15 Glu lines, 7 Cys lines and 7 EV lines), along with Wt (non-transgenic) plants.

To test the gene induction system, we isolated mitochondria from Wt plants and from all 22 transgenic Cys

and Glu lines and compared their AOX protein level using Western analyses. Fig. 1b shows a sample of the results. Mitochondria from Wt plants had relatively low levels of AOX protein and this level was not altered by a 24 h DEX treatment. The degree of AOX induction by DEX in any particular transgenic line correlated well with the level of GVG expression in that line. For example, while Cys10 and Cys13 (lines with high GVG expression; see later) displayed strong induction of AOX, Cys12 (with low GVG expression) gave little to no AOX induction (Fig. 1b). Similarly, Glu28 gave strong induction, while Glu15 did not. In all, 15 of the 22 lines gave strong AOX induction, while the remaining 7 lines (Cys12, Glu8, Glu12, Glu13, Glu15, Glu50, Glu57) gave little to no AOX induction (Fig. 1b and data not shown) and these same 7 lines also displayed the poorest GVG expression (see later). Taken together, these results confirm other studies indicating that the GVG system is effective at inducing gene expression in tobacco, that induction is dependent upon DEX, and that the level of induction correlates with GVG levels. In a few transgenic lines (e.g., Glu28, Fig. 1b), AOX levels in the absence of DEX were higher than in the Wt, suggesting some “leakiness” in the GVG expression system, perhaps due to position effects in the genome.

An aberrant phenotype associated with the GVG expression system in tobacco

We identified a number of Cys and Glu lines that, following DEX application and AOX induction, displayed a strong visible shoot phenotype. Some typical examples of this phenotype are shown in Fig. 2a. In particular, growth was strongly retarded and leaves became chlorotic within a few days. Also, the leaf surface became highly uneven and the leaves displayed a pronounced turgidity. A strong downward curling of leaves was another typical feature, as more readily viewed in Fig. 2b.

The severity of the above-described phenotype varied between lines, but was easily recognizable within 3 of 7 Cys lines and 4 of 15 Glu lines. However, this phenotype did not appear to be due to the induced expression of AOX, as the same phenotype was also seen in 2 of the 7 EV lines examined (Fig. 2a). In all cases, the phenotype was dependent upon the presence of DEX.

Northern blot analyses were used to determine the level of GVG transcript in all of the transgenic lines. Since we could not directly compare transcript level across all the lines simultaneously on a single RNA gel, we chose to analyze the lines in four groups. One group consisted of the Cys lines, two groups consisted of

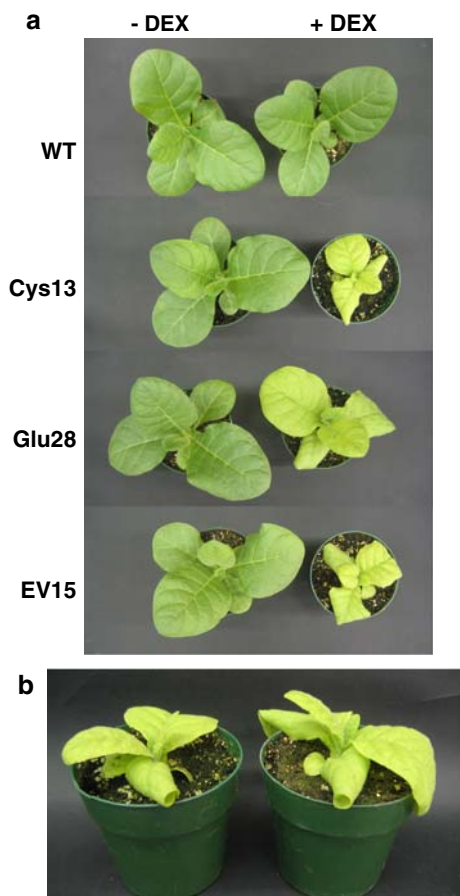


Fig. 2 Effect of DEX on the appearance of Wt and transgenic tobacco plants. **a** Plants on the right were sprayed once daily with 30 μM DEX for 6 days prior to photography. Plants on the left were sprayed in an identical manner except that the solution did not contain DEX. All plants were grown and maintained in the same growth chamber at an irradiance of approximately 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. **b** Close-up of two plant lines (EV15 on the left; Cys13 on the right) sprayed with 30 μM DEX for 6 days prior to photography. Note the dramatic downward curling of leaves

different Glu lines and another group consisted of EV lines. Fig. 3a shows the relative expression level for all lines within these four groups. GVG levels of lines within a group can thus be compared directly (since the levels were determined from the same Northern blots), while comparison across groups must be done more cautiously (since each group was analyzed on different Northern blots). As would be expected for gene expression being driven by the CaMV 35S promoter, the relative expression level data (calculated as indicated in the Fig. 3 legend) indicate that some lines within a group displayed much higher levels of GVG expression than other lines. In particular, note that all nine lines that showed the aberrant shoot phenotype (denoted by black bars in Fig. 3a) also displayed higher than average levels of GVG transcript (Fig. 3a).

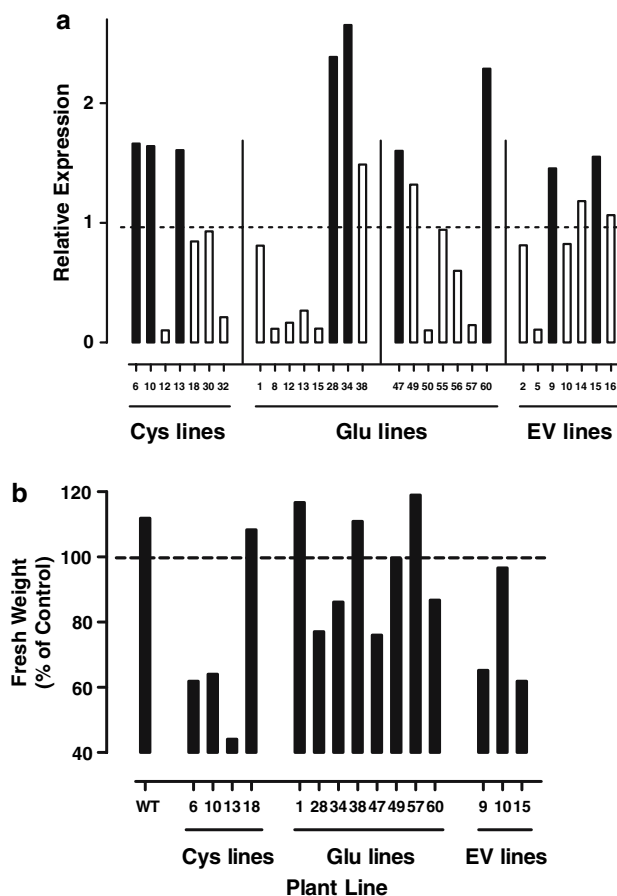


Fig. 3 a Relative expression of GVG in 29 different transgenic tobacco lines. Note that the lines are split into four groups with seven or eight transgenic lines within each group. Levels of GVG expression should only be directly compared between lines within the same group, since only these sets of transcripts were analyzed together by Northern analyses (see Results for more details). The four groups are delineated by the three vertical lines between data bars. For this analysis, all of the densitometer values of GVG transcript level for a particular blot were averaged. The densitometer value of each individual line was then divided by this average value to determine relative expression. Hence, lines with a relative expression value below 1 have lower than average GVG transcript levels (for the lines on that particular blot), while lines with a relative expression value above 1 have higher than average GVG transcript levels. The lines identified by black (rather than open) bars are those that display the strong aberrant shoot phenotype in the presence of DEX. **b** Fresh weight of WT and transgenic tobacco plants. Plants were sprayed with or without DEX for 6 days, as described in Fig. 2. For each plant line, data show the average fresh weight of three DEX-sprayed plants as a percentage of the average fresh weight of three plants sprayed without DEX

Figure 3b shows growth data for a subset of the Cys, Glu and EV lines, including the nine lines that showed the most noticeable shoot growth retardation after DEX treatment. Note that the growth of Wt plants and the remaining Cys, Glu and EV lines was not retarded by DEX treatment (Fig. 3b).

Light conditions affect the development of the aberrant shoot phenotype

In all of the experiments described above, plants were grown at an irradiance of approximately $400 \mu\text{mol m}^{-2} \text{s}^{-1}$. These growth conditions were kept constant both before and after spraying with DEX and resulted in the aberrant shoot phenotype described above. However, we found that the severity of the aberrant phenotype was reduced if, concurrent with the spraying of DEX, the plants were transferred to a lower irradiance of approximately $100 \mu\text{mol m}^{-2} \text{s}^{-1}$. For simplicity, we refer to those plants kept at the high irradiance in these experiments as HL plants and those transferred to the lower irradiance after DEX application as LL plants. Figure 4a shows some typical examples of how the aberrant phenotype could be partially negated by transfer to LL.

Given the ability of irradiance to influence development of the aberrant phenotype, we analyzed the transcript level of GVG in HL versus LL plants following DEX treatment. Interestingly, there was a small ($\sim 10\%$) but consistent and statistically significant decline in GVG transcript level in all transgenic lines following transfer to LL (Fig. 4b).

As a control, we analyzed the transcript level of the nuclear gene encoding FeSOD. FeSOD is a chloroplast-localized enzyme that converts superoxide to hydrogen peroxide and its expression has previously been shown to respond to changes in irradiance (Tsang et al. 1991). The absolute level of FeSOD transcript at a given irradiance was similar amongst the Wt and all transgenic lines analyzed (Fig. 4c). Also, the transcript level responded similarly in all lines to a change in irradiance. That is, upon transfer from the HL to LL condition, FeSOD transcript consistently declined (\sim twofold)(Fig. 4c).

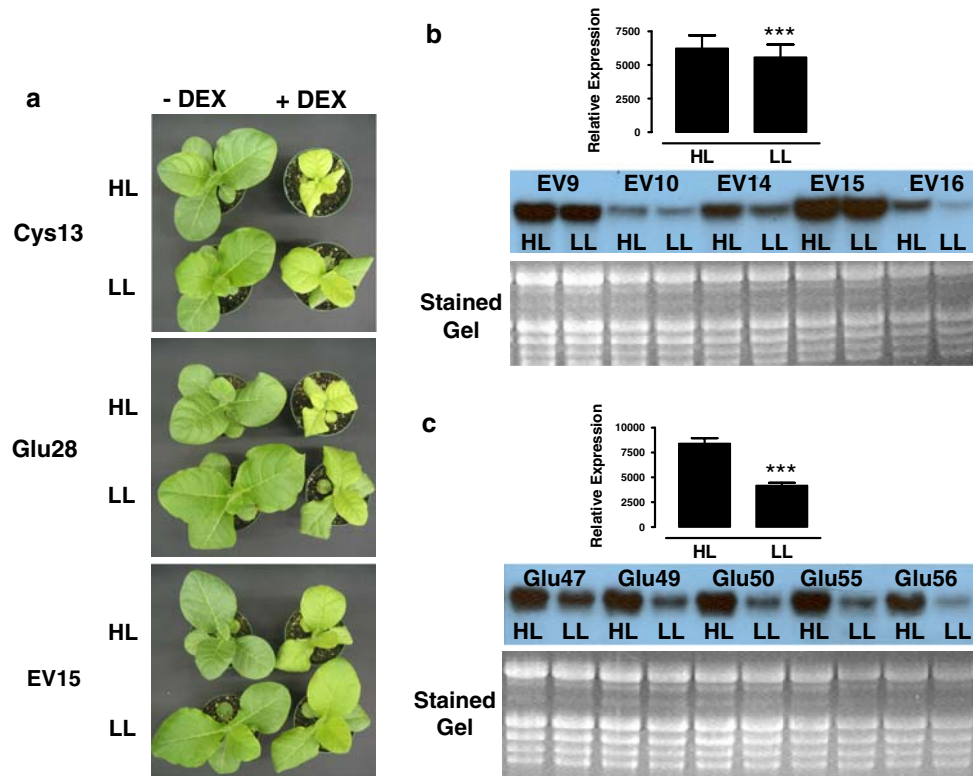


Fig. 4 a Effect of DEX and irradiance on the appearance of transgenic tobacco plants. Plants were sprayed with or without $30 \mu\text{M}$ DEX each day for 6 days prior to photography. Over this 6-day period, HL plants were kept at the same growth irradiance at which they were initially grown (approximately $400 \mu\text{mol m}^{-2} \text{s}^{-1}$), while LL plants were transferred to a lower irradiance (approximately $100 \mu\text{mol m}^{-2} \text{s}^{-1}$). Note that all three of these transgenic lines show an aberrant phenotype in the presence of DEX, but that the severity of this phenotype is reduced at LL. Gene transcript levels of GVG (**b**) and FeSOD (**c**) in tobacco plants at HL and LL. Chamber-grown tobacco plants were sprayed with $30 \mu\text{M}$ DEX, followed by incubation for 8 h at the same irradiance level

at which they were grown (HL) or at a lower irradiance (LL), prior to RNA isolation and Northern blot analysis. The data in (**b**) is the mean \pm SE from 22 transgenic lines with modest to high levels of GVG. The data in (**c**) is the mean \pm SE from both WT and 15 transgenic lines. For both GVG and FeSOD, all plants analyzed displayed lower levels of transcript at LL than HL and paired 2-tail *t* tests indicated a significance difference ($P < 0.0001$) between transcript level at HL versus LL. Also shown are representative Northern blots for some of the lines analyzed, as well as their corresponding ethidium bromide stained gels. *** indicates $P < 0.0001$

A molecular marker for the aberrant shoot phenotype

As shown earlier (Fig. 2b), one consistent aspect of the aberrant shoot phenotype in chamber-grown plants was a pronounced downward curling of leaves that appeared reminiscent of an ethylene-induced epinastic response. We therefore analyzed the transcript level of the nuclear gene encoding ACC oxidase, the enzyme that catalyzes the last step in ethylene biosynthesis. Strikingly, we found that all

nine of the transgenic lines, which displayed the strong aberrant phenotype (and hence also had high GVG levels) also displayed very high levels of ACC oxidase transcript at 8 h after DEX application in comparison to all other lines or WT plants (Fig. 5a). Also, at least one line (Glu49) that had not been visually identified to have the aberrant phenotype (although its GVG expression level was above average, Fig. 3a) displayed moderate induction of ACC oxidase (Fig. 5a).

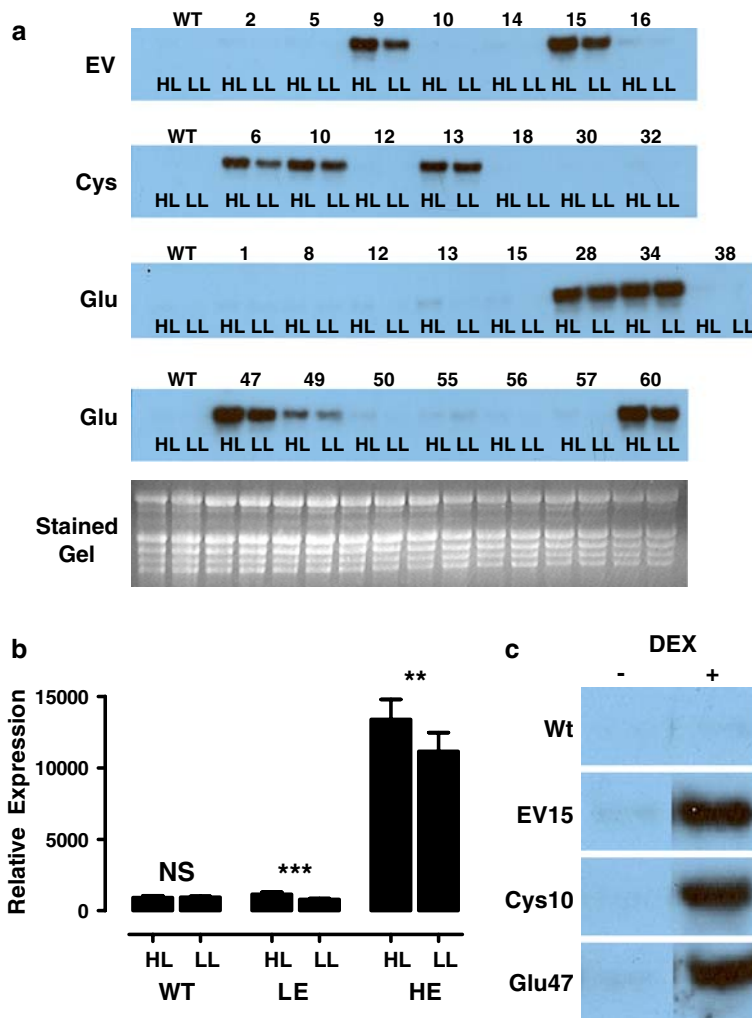


Fig. 5 a Northern blots showing ACC oxidase gene transcript level in Wt and transgenic tobacco plants. Chamber-grown tobacco plants were sprayed with 30 μM DEX, followed by incubation for 8 h at the same irradiance level (i.e., HL) or at a LL, prior to RNA isolation and Northern blot analysis. Note that a subset of the transgenic tobacco lines have very high ACC oxidase gene transcript levels in comparison to the remaining transgenic lines or WT tobacco. **b** A summary of ACC oxidase gene transcript level in WT and transgenic plants. Wt plants displayed low levels of ACC oxidase transcript in the presence of DEX and this level was not significantly impacted by irradiance level. Some transgenic plants in the presence of DEX had similar low levels of ACC

oxidase transcript as WT plants (denoted as *LE*, low expressors) but the level was still significantly lower at LL than HL ($***P < 0.001$). Some transgenic plants in the presence of DEX had much higher levels of ACC oxidase transcript than WT plants (denoted as *HE*, high expressors) and the level was significantly lower at LL than HL ($**P < 0.01$). Data are mean ± SE and were analyzed using a paired 2-tail *t* test. **c** Northern analysis of ACC oxidase gene expression in Wt and transgenic tobacco plants left untreated or treated with 30 μM DEX for 8 h. Plants were kept under their usual growth irradiance (i.e., HL) after treatment with DEX

As we found for GVG, the transcript level of ACC oxidase declined slightly when plants were kept at LL versus HL, following DEX treatment (Fig. 5b). This decline was significant regardless of whether the transgenic lines were those that expressed low levels of ACC oxidase (LE lines) or high levels of ACC oxidase (HE lines). Wt plants had low levels of ACC oxidase expression that was not significantly impacted by irradiance level (Fig. 5b).

To confirm that the high level of ACC oxidase gene transcript seen in some transgenic lines (Fig. 5a) was indeed DEX-dependent, we compared the transcript level of untreated EV15, Cys10, Glu47 and WT plants. Fig. 5c shows that the transcript level was low in all untreated plants (both transgenic and WT), but that after 8 h of DEX treatment, the transcript level was high in the transgenic lines.

Disparate growth conditions can result in disparate aberrant growth phenotypes

As another independent test of whether the GVG expression system caused side effects, a selection of the transgenic plants were germinated and grown at room temperature on agar plates (with or without DEX) and under continuous low fluorescent light ($\sim 40 \mu\text{mol m}^{-2} \text{s}^{-1}$). Under these disparate growth conditions and developmental stage from all those experiments described above, we did not see as dramatic an effect of DEX on shoot growth or appearance (data not shown). However, we found that in those same transgenic lines previously found to display the aberrant shoot phenotype and to express the highest levels of GVG, root growth was now being strongly inhibited by DEX (Fig. 6). Again, this aberrant phenotype was also being displayed by the EV lines that contained high levels of GVG. On the other hand, WT seedlings or transgenic seedlings containing low to moderate GVG levels did not exhibit this strong inhibition of root growth. In fact, Wt root length was slightly enhanced by the DEX treatment (Fig. 6).

Discussion

The GVG gene expression system has been shown to cause growth defects and/or other aberrant phenotypes in a wide taxonomic range of plants including the eudicot *Arabidopsis thaliana* (Kang et al. 1999), the monocot rice (Ouwerkerk et al. 2001), the model legume *Lotus japonicus* (Andersen et al. 2003) and the gymnosperm *Pinus taeda* (Tang and Newton 2004). These studies have highlighted the importance of using empty

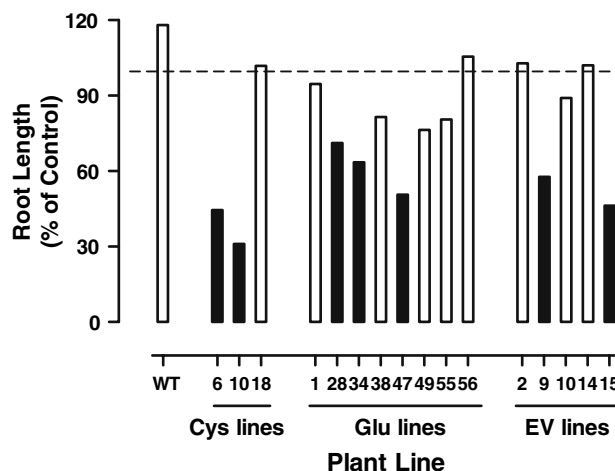


Fig. 6 Effect of DEX on root growth of WT and transgenic tobacco seedlings germinated and grown on agar medium for 23 days. For each plant line, data show the average root length of ~ 100 seedlings germinated in the presence of $30 \mu\text{M}$ DEX as a percentage of the average root length of ~ 100 seedlings germinated in the absence of DEX. The lines identified by *black* (rather than open) bars are those that were previously found to display the strong aberrant shoot phenotype in the presence of DEX and to display high levels of GVG expression. For each plant line, data are pooled from multiple independent experiments and agar plates, all of which showed similar results

vector lines and other strategies to ensure that biological responses being studied are due to the induced expression of one's gene of interest and not due to the inducible expression system itself. Our results suggest that extreme care needs to be taken when using the GVG expression system in tobacco, as well. In our experiments, approximately 30% of the transgenic plants produced displayed serious side effects attributable to the gene expression system. This is significant because, up until now, the system has been largely accepted to be a relatively problem-free system for use in *N. tabacum* (Moore et al. 2006) and it continues to be widely used in this species (eg. Nara et al. 2000; Shen 2001; Yang et al. 2001; Geelen et al. 2002; Barrero et al. 2002; Barrero et al. 2003; Grémillon et al. 2004; Shen and Meyer 2004; Ogawa et al. 2005; Yang et al. 2005; Clément et al. 2006) and other *Nicotiana* species (e.g., Qin and Zeevaart 2002; Mori et al. 2001). However, none of the GVG expression system-dependent abnormalities seen in other species have been previously reported for tobacco.

Our results strongly suggest that the aberrant effects of the GVG expression system in tobacco are due to non-specific effects of the constitutively expressed chimeric GVG transcription factor. This is based on the observation that only those transgenic lines showing higher than average levels of GVG transcript are susceptible to these aberrant effects. It is also clear that

the aberrant phenotype only occurs in the presence of DEX, indicating that GVG levels need not only be high, but also that GVG must be in its activated state as well. These results correspond with those published for other species (Kang et al. 1999; Andersen et al. 2003). DEX itself is not responsible for the aberrant phenotype; otherwise all plants sprayed with DEX would be expected to display the aberrant phenotype. Other studies have shown that treatment of WT tobacco plants for four weeks with up to 60 μM DEX had no impact on growth and development (Moore et al. 2006). We also saw no aberrant effects of 30 μM DEX on growth or gene expression in our WT plants (Figs. 1, 2, 3, 5, 6).

Our results also suggest that the chimeric GVG transcription factor has some unknown strong impact on biosynthetic or signaling pathways related to ethylene. This is based on our observation that plants expressing high GVG display a rapid and strong induction of ACC oxidase gene expression in response to DEX. ACC oxidase catalyzes the conversion of ACC to ethylene and is considered an important rate-limiting enzyme in the ethylene biosynthetic pathway. Consistent with this observation, plants inducing ACC oxidase expression showed a strong leaf epinasty. Hormonal relations associated with leaf epinastic responses have been studied in some detail and both ethylene and auxin appear to interact during this developmental response, as they do in numerous other responses (Hayes 1981; Romano et al. 1993; Keller and Van Volkenburgh 1997; Peck and Kende 1995; Stepanova and Alonso 2005). Interestingly, studies showing aberrant effects of the GVG expression system in other species have also implicated hormonal effects. Aberrant effects of the GVG expression system in *L. japonicus* were speculated to arise due to effects on auxin signaling (Andersen et al. 2003).

The GVG expression system has been described to cause shoot growth defects in *Arabidopsis* and, in this case, the growth defect was accompanied by an increased expression of the defense-related gene PDF1.2, but only after 48 h (Kang et al. 1999). Interestingly, the expression of PDF1.2 is also strongly induced (and in a similar 48 h time frame) by ethylene treatment (Penninckx et al. 1998). This indicates that the growth defect described earlier in *Arabidopsis* may also be related to ethylene.

We found that the susceptibility of plants to the aberrant shoot phenotype was highly dependent upon growth conditions. The degree of growth retardation and chlorosis could be partially reduced by low light, and the increase in ACC oxidase gene expression was not as severe. Hence, lower light reduced the

susceptibility of plants to the side effects of high GVG expression. This may be at least partly due to the apparent slight drop in CaMV-driven GVG expression that was noted at low light.

For many of the reports in which the GVG expression system has been used in tobacco (including the original work of Aoyama and Chua 1997), experiments were done in tissue culture, suspension culture or with seedlings grown on agar plates. The low light conditions typical of such experiments may provide some explanation why the aberrant effects of the GVG system in this species have not been reported before. Indeed, when we germinated and grew plants on agar plates, we saw no noticeable shoot phenotype associated with the combination of high GVG expression and the presence of DEX. However, under these conditions, another aberrant phenotype (a strong inhibition of root growth) was clearly attributable to the GVG expression system.

Our results indicate that great care should be taken to ensure the usefulness of the GVG expression system in tobacco. In particular, multiple empty vector lines (we suggest five to ten) need to be analyzed to confirm that the biological effects being attributed to one's gene of interest are not due to the expression system itself. Further, since both characteristics of and susceptibility to the GVG side effects appear to be dependent upon growth conditions (and possibly developmental stage as well), parallel experiments with empty vector lines need to be included for all of the conditions under which one's gene of interest is being evaluated. As demonstrated elsewhere (Ouwkerk et al. 2001), the concentration of DEX is another factor that may impact susceptibility to the side effects.

Further, we have identified an early, sensitive and robust molecular marker (ACC oxidase gene expression) for the aberrant shoot phenotype in tobacco. This provides a means to identify problem plants with much more precision than can be afforded by relying solely on macroscopic features (e.g., growth retardation, chlorosis). For example, while we saw no obvious visible shoot phenotype in Glu49 (although its GVG level was quite high), there was a moderate induction of ACC oxidase after DEX, suggesting that this plant is at a threshold that limits its usefulness. The aberrant side effects of the GVG expression system clearly call for extra scrutiny in the selection of plants for study. Nonetheless, we were able to identify several plants (e.g., Cys18, Glu1, Glu56), whose GVG levels were high enough to give good induction of our protein of interest (AOX), yet showed no change in ACC oxidase gene expression above that seen in Wt plants. This indicates that the threshold level of GVG required for

good transgene induction in tobacco is still below that which causes the aberrant shoot phenotype, unlike the situation in *L. japonicus* (Andersen et al. 2003).

There are some cases in which the DEX-induction system has been used in tobacco for studies relating directly to ethylene biology (e.g., Ogawa et al. 2005). Our results suggest that this should either be avoided altogether or that extreme care will need to be exercised for such studies. In such cases, a new DEX-inducible gene expression system recently described for tobacco (Samalova et al. 2005) may provide a practical alternative.

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