1 **Inducible double-stranded RNA expression activates reversible transcript**

2 **turnover and stable translational suppression of a target gene in transgenic**

3 **tobacco**

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9   A binary vector amenable to high-throughput cloning was constructed for

10 ethanol-inducible expression of double-stranded RNA (dsRNA) in plants.   Silencing

11 of a transgene encoding β-glucuronidase (*GUS*) was then examined at RNA and

12 protein levels in tobacco.   Transient gene silencing could be effectively achieved in

13 plants with higher expression levels of *alcR* (the ethanol sensor) after single

14 application of 1% ethanol (v/v) through root drenching.   GUS activities showed

15 more dramatic pattern of loss and recovery in young leaves than in older leaves.

16 Repeated ethanol treatment resulted in extended gene suppression and increased loss

17 of GUS activities.   Interestingly, recovery of GUS transcript level is dramatically

18 earlier than that of GUS protein levels as measured by enzyme assays.   These

19 observations indicate that dsRNA-mediated gene silencing may occur through more

20 stable translational inhibition in addition to reversible targeted RNA degradation.

21

1   *Keywords*: dsRNA, gene silencing, ethanol induction, GUS

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3   *Abbreviations*: GUS, β-glucuronidase; dsRNA, double-stranded RNA; PTGS,

4   post-transcriptional gene silencing

1. Introduction

Double-stranded RNA (dsRNA)-mediated post-transcriptional gene silencing (PTGS) has emerged as an effective approach to investigate gene functions. In plants, dsRNA-mediated PTGS is commonly achieved by stable transformation of an intron-containing self-complementary "hairpin" RNA (hpRNA) construct. The presence of a spliceable intron appears to enhance the formation of dsRNA and hence the targeted mRNA degradation [1-2]. Constitutive expression of dsRNA using the cauliflower mosaic virus 35S promoter could achieve PTGS with almost 100% efficiency when directed against viral or endogenous genes in plants [1]. However, this approach could limit the study of essential genes since recovery of transgenic lines would become difficult. An inducible system is therefore highly desirable for control of gene silencing in plants. The *alc* regulon of *Aspergillus nidulans* was recently introduced to plants to induce gene expression using ethanol with negligible basal activity [3-4]. The *alc* regulon contains two components: *alcR*, a gene encoding a transcription factor which is the ethanol sensor, and *palcA*, an ALCR-responsive promoter from the alcohol dehydrogenase I gene. Binding of ethanol to ALCR changes its conformation and initiates subsequent binding of the ALCR-ethanol complex to *palcA*, resulting in transcriptional activation [5-6]. The *alc* regulon could also be induced by related chemicals such as acetaldehyde [7].

In this study, we constructed a new binary vector by placing an hpRNA-encoding unit derived from the pHellsgate2 vector [2] under the control of the alcohol-inducible system. The target gene hpRNA construct can be generated in a single step with a

3

1 PCR product through recombination cloning [2].   Characterization of ethanol

2 inducible gene silencing was carried out using a transgene target encoding

3 β-glucuronidase (*GUS*) because of its common application as a facile expression

4 marker in transgenic plants [3-4, 8-9].   This work defines parameters important for

5 the analysis of gene function at both the RNA and protein levels using our inducible

6 dsRNA system.   The relevance of our findings to the mechanisms of PTGS in plants

7 is discussed.

8

2. Materials and methods

10 *2.1 Vector construction*

11   Plasmids for the alcohol induction system (*alcR* gene and *palcA*) were kindly

12 provided by A.B. Tomsett (University of Liverpool, UK).   The *alcR* coding region

13 was cloned into 103c-SK (E. Lam, unpublished), an over-expression vector

14 containing the CaMV 35S promoter and the nopaline synthase 3'-terminator (nos3').

15 The 35S-alcR-nos3' fragment was then inserted into a pCambia 1300 binary vector

16 (CAMBIA, Australia) cloned with *palcA*.   The hpRNA-encoding unit containing two

17 oppositely oriented recombination sequences of *attP1* and *attP2* and the octopine

18 synthetase 3'-terminator (ocs3') was removed from the pHellsgate2 plasmid (P.

19 Waterhouse, CSIRO Plant Industries, Australia) and inserted downstream of *palcA*.

20 The final binary vector was named pMW4 (Fig. 1), with hygromycin resistance as the

21 selection marker.

1   A *GUS* gene-specific fragment (from +993 to +1393 relative to the translation

2   start site) was PCR-amplified using primers: Forward, *attB1-GUS*-F (5'-GGG GAC

3   AAG TTT GTA CAA AAA AGC AGG CT CCC TTA CGC TGA AGA GAT GC-3');

4   Reverse, *attB2-GUS*-R (5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GT

5   GGC ACA GCA CAT CAA AGA GA-3') with the GUS-coding sequences underlined.

6   The alcohol inducible *GUS* dsRNA vector (pMW4G) was generated by recombination

7   of the *attB1* and *attB2* sites (flanking the PCR product) with the *attP1* and *attP2* sites

8   in pMW4 (Fig. 1) using BP clonase (Invitrogen) following the manufacturer's

9   instructions.

10   *2.2 Plant transformation, growth and maintenance*

11   A transgenic tobacco plant (*cv. Samsun NN*) carrying a *GUS* gene driven by the

12   CaMV 35S promoter with a kanamycin selection marker (Fig. 1; pEL-104B, E. Lam,

13   unpublished) was used for *Agrobacterium*-mediated transformation.

14   pMW4G-containing plants were regenerated on $1\times$ Murashige and Skoog (MS) agar

15   containing hygromycin B (50 µg ml^{-1}).   When transgenic plants grew up to 4 cm,

16   they were transferred to $1\times$ MS agar containing hygromycin B (25 µg ml^{-1}).   After

17   two weeks, the plants were transferred to soil and grown in a greenhouse at

18   22°C/18°C (day/night) and 16h/8h day/night photoperiod.

19   *2.3 Ethanol induction and tissue collection*

20   Transgenic tobacco plants (T1) were selected on hygromycin and confirmed for

21   GUS enzyme activities by staining with X-GLUC (Clontech).   One to two-month old

1   T1 plants were grown in 4-L soil and treated with 600 ml of ethanol at the appropriate

2   concentrations (v/v) by root drenching.   Application time was in the morning for

3   single ethanol induction.   For repeated applications, ethanol was added in the

4   morning at 24 h intervals for 3 d.   Leaf tissues were collected from at least three

5   individual T1 plants at various time intervals.   Tissues from each time interval were

6   pooled together for analyses and individual experiments were repeated twice.

7   *2.4 RNA experiments*

8   For total RNA preparations, leaf tissues were grinded in liquid nitrogen and

9   extracted with Trizol reagent (Invitrogen) following the manufacturer's instruction.

10   For northern analyses, denatured RNA samples were separated on a 1.5% agarose

11   formaldehyde gel, transferred to Hybond-N+ membranes (Amersham Bioscience),

12   and hybridized with a ^{32}P-dCTP-labeled *GUS* specific probe.   Quantitative analysis

13   of radio-labeled signals was carried out using the software ImageQuant 4.0.1

14   (BioRad).   Each data point was normalized based on the signal detected by a

15   ^{32}P-dCTP-labeled probe specific to the tobacco 18S rRNA in the corresponding

16   sample.   Detection of small interfering RNA (siRNA) was performed essentially as

17   described [10]. ^{32}P-UTP-labelled *GUS* riboprobe generated using the Riboprobe *in*

18   *vitro* transcription system (Promega) was used for hybridization.

19   *2.5 GUS enzyme assays*

20   Leaf tissues were homogenized in protein extraction buffer (50 mM $Na_2H_2PO_4$,

21   10 mM EDTA, 0.1% triton X-100, and 1.0 g L^{-1} sarcosyl) and protein concentrations

1   measured as described previously [10].   For GUS activity determination, a

2   fluorimetric assay was conducted using the substrate 4-methylumbelliferyl

3   β-D-glucuronidase essentially as described [11].   Reaction products were quantified,

4   using 4-methylumbelliferone (4-MU) as a standard, by measurements at the 360 nm

5   excitation and 460 nm emission wavelengths in a microtiter plate reader.

6

7   **3. Results**

8   *3.1 Ethanol-inducible GUS silencing*

9   A transgenic tobacco line with constitutive *GUS* expression was transformed with

10   the alcohol inducible *GUS* dsRNA vector (pMW4G) under hygromycin selection.

11   A total of 19 tobacco plants were regenerated and leaf tissues from each primary

12   transformant (4-5 leaf stage) were collected and analyzed for *alcR* gene expression by

13   northern blots (data not shown).   To investigate whether *alcR* expression level would

14   affect the degree of gene silencing, T1 lines of a low *alcR* expressor (LT1) and a high

15   *alcR* expressor (HT1) were treated with 1% ethanol (v/v).   Northern experiments

16   were then performed with RNA samples collected from leaf tissues at different time

17   intervals.   Quantitative analysis of *alcR* northern signals showed that the expression

18   level in the HT1 line was 2.7 times of that in the LT1 line (Fig. 2).   Near-complete

19   *GUS* silencing was detected 24 h after ethanol treatment in both lines.   A more rapid

20   inducible response was found in the HT1 line with over 90% gene silencing 6 h after

21   treatment, compared to only 60% gene silencing detected in the LT1 line (Fig. 2).

1   To characterize the dose-response behavior of our system, T1 plants of the HT1

2   line were treated with single applications of different concentrations (v/v) of ethanol.

3   Following treatment, all plants showed maximum levels of *GUS* silencing after 12-24

4   h and near-complete recovery of *GUS* expression occurred after 48 h (Fig. 3).

5   However, only 50% gene silencing was achieved in plants treated with 0.1 % (v/v)

6   ethanol.   Similar patterns of gene silencing after treatment with 1% or 2% (v/v)

7   ethanol were observed, suggesting saturation of the response at around 1% (v/v)

8   ethanol.

9   *3.2 Suppression of GUS enzyme activities*

10   The effects of ethanol inducible gene silencing on GUS protein levels were

11   investigated among leaf tissues of different stages (Fig. 4A) in the HT1 T1 plants

12   following 1% ethanol (v/v) treatment.   Similar patterns of *GUS* silencing were

13   observed in young (zone 1), mature (zone 2), and older (zone 3) leaves (Fig. 4A).

14   Using enzyme assays as an indirect measurement for GUS protein levels, suppression

15   of enzyme activity was found to be less dramatic in older leaves compared to younger

16   leaves (Fig. 4B).   For example, leaves in zone 3 showed only 30% decrease in GUS

17   activity compared to an 80% reduction in zone 1 24 h after ethanol treatment, during

18   which *GUS* gene expression was largely silenced.   In all cases, the enzyme activities

19   started to recover as accumulation of GUS transcripts resumed (Fig. 4).

20   Older tissues were expected to accumulate abundant levels of GUS enzyme

21   which is a stable protein (Jefferson et al., 1987).   The initial GUS activities were at

22   least two-fold higher in zone 3 than in zone 1 (data not shown).   Thus, the enzyme

1   activity might not be suppressed significantly in old tissue when the transcript level

2   was silenced transiently.   In an attempt to achieve extended gene silencing, HT1

3   plants were treated with 1% ethanol (v/v) at 24 h intervals for 3 days.   As shown in

4   Fig. 5A-B, repeated treatments of ethanol maintained *GUS* gene silencing for 96 h,

5   which was 48 h after the final treatment.   The HT1 plants were also treated with 3%

6   ethanol (v/v), which showed a similar pattern of gene silencing and recovery to those

7   treated with 1% ethanol (v/v).   Thus, the extension of gene silencing likely resulted

8   from repeated treatments instead of an increase in the total amount of ethanol applied.

9   GUS activities were then determined in older leaf tissues collected from plants

10   after different treatments.   Single treatment of either 1% or 3% (v/v) ethanol resulted

11   in similar patterns of change in GUS activities, with maximum suppression by about

12   30% detected after 24-48 h (Fig. 5C).   In contrast, a gradual decline in GUS

13   activities extending to 144 h after the first treatment was observed in plants with

14   repeated treatments.   In addition, suppression of GUS activity by 95% was detected

15   in these plants at 144 h when *GUS* transcripts had returned to 80% of control level for

16   about 2 days (Fig. 5B-C).   This observation suggests that translation of GUS protein

17   could be stably suppressed by extended presence of target gene dsRNAs in the plants

18   upon repeated ethanol treatments.

19

20   **4. Discussion**

21   A general method for inducible expression of dsRNA-mediated gene silencing in

1   plants was first reported with the use of an irreversible estradiol-inducible

2   recombination approach [12].   Using our construct, we demonstrated that target gene

3   silencing occurred in a reversible manner following ethanol induction.   Ethanol was

4   likely to be lost rapidly through evaporation or plant metabolism.   In addition, the

5   dsRNAs generated may silence the target gene without epigenetic or

6   self-amplification components that would have maintained PTGS after the induction

7   system is turned-off.   The reversible nature of our system should allow temporal

8   characterization of gene functions without suppression of a target gene throughout the

9   plant life cycle.   Efficient gene silencing could be achieved in transgenic plants with

10   high levels of *alcR* expression using 1% (v/v) ethanol treatment.   In addition, the

11   incorporated recombination cloning system is useful for large-scale generation of

12   plant transformation constructs in a single cloning step [2].

13   Gene silencing approaches rarely achieve complete suppression at the protein

14   level in general.   However, gene functions can be determined if dramatic changes in

15   the protein level can be obtained.   Achieving this objective with an inducible PTGS

16   approach would depend on the target protein level and its stability in tissues from

17   different developmental stages.   The *35S-GUS* expression cassette represents a good

18   system to establish the protocol necessary to silence a strongly expressed gene with a

19   stable protein product.   Transient gene silencing was apparently not sufficient to

20   suppress effectively the more abundant levels of stable GUS protein in older leaf

21   tissues (Fig. 4B).   Instead, an eventual decline of the enzyme activities was detected

22   only when extended gene silencing was achieved by repeated ethanol treatments (Fig.

1   5C), presumably accompanied by a slow and gradual turnover of the pre-existing

2   GUS protein.   A similar ethanol inducible approach was used recently to silence

3   chlorophyll biosynthesis genes in transgenic tobaccos [13].   In that study, the loss of

4   chlorophyll pigments was observed in young leaves but not in mature leaves

5   following ethanol treatment.   However, this difference in silencing phenotypes was

6   not characterized in detail at the RNA and protein levels.   Based on our present

7   results, their target proteins were likely to be more susceptible to the inducible

8   suppression in young tissues, giving rise to the developmental stage-specific

9   phenotype observed.

10   In this study, repeated induction treatments resulted in suppression effects that

11   could not be achieved simply by increased ethanol doses.   Duration of gene silencing

12   was extended following three applications of 1% (v/v) ethanol at 24 h intervals.

13   Interestingly, *GUS* transcripts did not start to re-accumulate until 72 h after the final

14   treatment (Fig. 5A-B).   This is in contrast to a single application in which gene

15   expression resumed by 48 h, irrespective of the concentration used (Fig. 3).   In

16   addition, the persistent loss of GUS activity when gene expression had largely

17   returned to pre-treatment levels was unexpected (Fig. 5).   Re-accumulation of *GUS*

18   transcripts indicated that dsRNA-mediated target mRNA degradation was essentially

19   turned-off.   However, the recovered transcript apparently did not result in significant

20   amount of GUS protein synthesis detectable by our assays, strongly suggesting that a

21   translational suppression component is associated with the dsRNA-mediated PTGS.

22   In this connection, the *Arabidopsis* microRNA miRNA172 was recently shown to

11

regulate *APETALA2* expression during floral development through translational

inhibition [14].   Our results suggest a common dsRNA signal could generate distinct

silencing activities that target specific RNA turnover and translational arrest

concomitantly.   For sustained gene silencing and protein suppression, the total

amount of ethanol added is obviously not critical compared to repeated treatments in

our experiments.   Thus, these phenomena are likely to result from prolonged dsRNA

exposure and/or a higher threshold level of dsRNA under persistent induction.

Closer examinations of our novel observations may reveal additional appreciation of

the complexities of dsRNA-mediated PTGS.

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FIGURE LEGENDS

2 Fig. 1. (A) Ethanol inducible dsRNA vector.   In pMW4, *alcR* is driven by the CaMV

3 35S promoter.   The hpRNA-encoding unit derived from pHellsgate2 with oppositely

4 oriented recombination sequences (*attP1-attP2*) was cloned downstream of the palcA

5 promoter.   To construct pMW4G, *GUS* PCR product with *attB1* and *attB2* flanking

6 sequences entered pMW4 at the *attP1* and *attP2* sites, respectively, through

7 recombination.   Two new sites were then generated: *attL1* and *attL2* in the vector;

8 *attR1* and *attR2* flanking the fragment excised out from the vector.   *ccdB*, a bacterial

9 lethal gene allowing positive selection of the desired construct; i, intron; *Hyg^r*,

10 hygromycin resistance. (B) GUS expression vector in the tobacco line used for

11 pMW4G transformation. *pnos*, nopaline synthase promoter.

12
13 Fig. 2.   Northern analysis of inducible *GUS* gene silencing in LT1 and HT1 lines.

14 T1 plants were induced with 1% ethanol (v/v) at time 0.   Northern blot signals (A)

15 were quantified by image analysis for comparison of gene expression levels (B).

16 The average level of *alcR* expression in HT1 plants was estimated to be 2.7 folds of

17 that in LT1 plants.   Expression levels of *GUS* gene at different time points were

18 normalized using the corresponding 18S rRNA signals and were expressed as

19 percentages of the initial levels.

20

21 Fig. 3.   *GUS* gene silencing following treatments with different ethanol

22 concentrations.   HT1 T1 plants were treated with single application of the indicated

23 ethanol doses (v/v) at time 0.   Northern signals of *GUS* transcripts at different time

24 points were normalized using the corresponding 18S rRNA signals and were

25 expressed as percentages of the initial levels.

26

1   Fig. 4.   Suppression of GUS enzyme activities in leaf tissues of different stages.   A.

2   Leaf tissues (8-10 leaf stage) were collected in the indicated zones for RNA and

3   protein extractions.   Northern signals of *GUS* transcripts at different time points

4   were normalized using the corresponding 18S rRNA signals and were expressed as

5   percentages of the initial levels.   B. GUS enzyme activities in different tissues were

6   expressed as percentages of the initial levels.

7

8   Fig. 5.   Silencing of *GUS* expression and suppression of GUS activity upon repeated

9   ethanol treatments.   Plants were treated with repeated applications (3×) of 1%

10   ethanol (v/v) at the indicated time points (arrowheads) or single applications (1×) of

11   ethanol (1% or 3%, v/v) at time 0.   Leaf samples were collected from zone 3 (Fig.

12   4A) for RNA and protein extractions.   A, B. Northern signals of *GUS* transcripts at

13   different time points were normalized using the corresponding 18S rRNA signals and

14   were expressed as percentages of the initial levels.   C. Time course analysis of GUS

15   activities in plants under different ethanol treatments.   Enzyme activities were

16   expressed as percentages of initial levels.   Two independent protein extracts and

17   assays were performed for the final 3 time points in the repeated ethanol treatments to
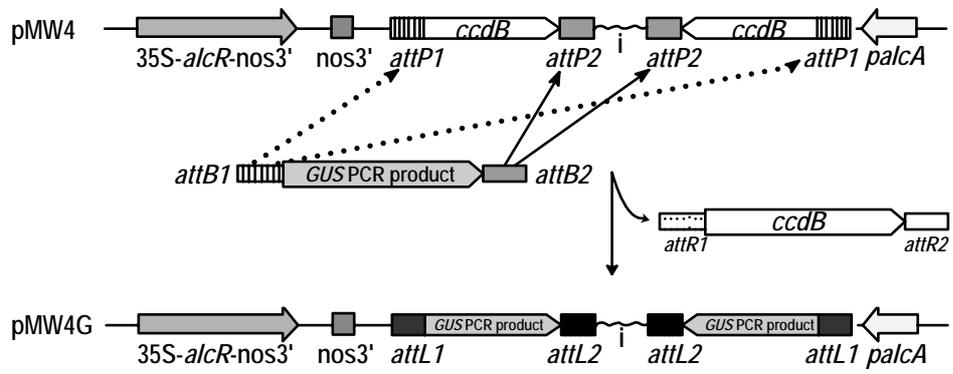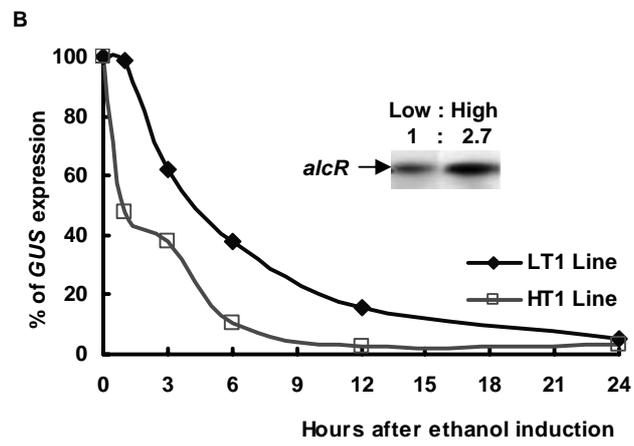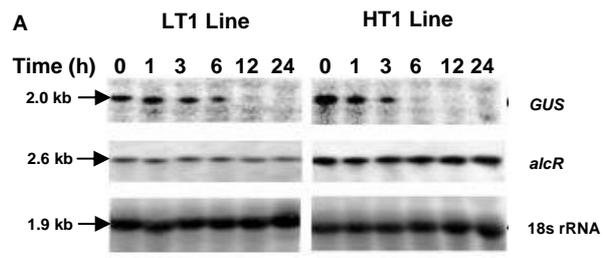
18   assure reproducibility.

Fig 1

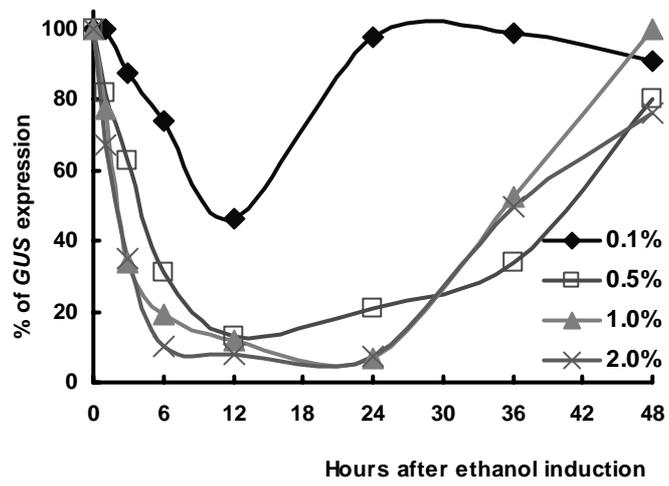**A**

LT1 Line   HT1 Line

Time (h)   0 1 3 6 12 24   0 1 3 6 12 24

2.0 kb →   *GUS*

2.6 kb →   *alcR*

1.9 kb →   18s rRNA

B

Low : High
1 : 2.7

alcR →

♦ LT1 Line
□ HT1 Line

% of *GUS* expression

Hours after ethanol induction

Fig 2

Fig 3

Fig 4

A

1% Ethanol, 1X

Time (h)   0   24 48 72 96 120 144

GUS 2.0Kb→

18S rRNA →

3% Ethanol, 1X

Time (h)   0   24 48 72 96 120

GUS 2.0Kb→

18S rRNA →

1% Ethanol, 3X

Time (h)   0   24 48 72 96 120 144

GUS 2.0Kb→

18S rRNA →

B

% of *GUS* expression

100
80
60
40
20

0   24   48   72   96   120 144

C

% of GUS activity

100
80
60
40
20
0

■ 1% Ethanol, 3X
▲ 1% Ethanol, 1X
□ 3% Ethanol, 1X

0   24   48   72   96   120 144

Hours after first ethanol application

Fig 5