Plant Cell Rep (2006) 25: 660–667
DOI 10.1007/s00299-005-0112-3

GENETIC TRANSFORMATION AND HYBRIDIZATION

S. A. Filichkin · R. Meilan · V. B. Busov · C. Ma · A. M. Brunner · S. H. Strauss

Alcohol-inducible gene expression in transgenic *Populus*

Received: 3 August 2005 / Revised: 19 October 2005 / Accepted: 20 November 2005 / Published online: 23 February 2006

Abstract We tested the efficiency and optimized the conditions for controlled alcohol-inducible transgene expression in *Populus* using *gus* as a reporter gene. Specificity of induction, efficiency in different organs, effect of three chemical inducers, and induction methods were tested using up to 10 independent transgenic events generated in two different *Populus* genotypes. The optimal inducer concentration and the duration of induction period were determined in dose–response and in time–course experiments. Under *in vitro* conditions, β-glucuronidase (GUS) induction was efficient both in the aerial parts and in the roots of regenerated plantlets. Among the chemical inducers tested, ethanol was the most effective activator with no apparent phytotoxicity when concentrations were at or below 2%. After 5 days of treatment, fluorometrically-determined the GUS activity could be detected when inducing with ethanol at concentrations as low as 0.5%. Prolonged induction by ethanol vapors significantly increased the GUS activity in leaves from both the tissue culture plants and greenhouse-grown plants.

Keywords Poplar · Aspen · Cottonwood · *alc* system · Functional genomics

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00299-005-0112-3

Communicated by D.A. Somers

S. A. Filichkin · C. Ma · A. M. Brunner · S. H. Strauss (✉)
Department of Forest Science, Oregon State University, Corvallis, OR, 97331
e-mail: steve.strauss@oregonstate.edu
Tel.: +1-541-737-6578
Fax: +1-541-737-1393

R. Meilan
Department of Forestry and Natural Resources, Purdue University,
West Lafayette, IN, 47907, USA

V. B. Busov
School of Forest Resources and Environmental Science, Michigan Technological University,
Houghton, MI, 49931, USA

Abbreviations Ac: Acetaldehyde · 2-B: 2-Butanone · BAP: 6-Benzylaminopurine · CaMV: Cauliflower mosaic virus · CIM: Callus-induction medium · dpi: Days post-induction · IBA: Indole-3-butyric acid · IM: *Agrobacterium*-induction medium · LB: Luria-Bertani medium · MS: Murashige and Skoog medium · RCF: Relative centrifugal force · SIM: Shoot-induction medium · TDZ: Thidiazuron · *uidA*: β-Glucuronidase, *gus* reporter gene · X-Gluc: 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Introduction

Modifying endogene expression is an important tool for functional genomics of plants. However, the constitutive expression or silencing of a target gene often leads to pleiotropic effects, making it difficult to directly relate gene function with an observed phenotype. Developmental regulatory genes are particularly difficult to study because their constitutive over expression or silencing often interferes with the recovery of viable transgenic plants. To overcome these limitations, several chemically inducible gene-switch systems have been recently developed and tested in model plants (reviewed in Padidam 2003).

An alcohol-responsive system employing components of the *alc*-inducible regulon of the fungus *Aspergillus nidulans* was effective in a wide range of model and crop plants (Caddick et al. 1998; Roslan et al. 2001; Sweetman et al. 2002; Deveaux et al. 2003; Schaarschmidt et al. 2004; Garoosi et al. 2005). The *alcR* gene encodes a protein that regulates transcriptional activation of the *alc*-inducible regulon, the product of which is required for the oxidation of ethanol (Flipphi et al. 2001). The alcohol sensor protein, AlcR, is a DNA-binding transcription factor (Mathieu et al. 2000). In the presence of inducers such as ethanol or aldehydes, AlcR activates the transcription of alcohol dehydrogenase by binding to a specific motif in the *alcA* promoter (Kulmburg et al. 1992). A transcriptional fusion of the *alcA*

promoter to a reporter gene activates the expression of the reporter upon treatment with an appropriate inducing agent.

Using a *35S*::*alcR-alcA*::*uidA* cassette, AlcR was reported to be an effective temporal and spatial regulator of *uidA* (*gus*) gene expression in *Arabidopsis* (Roslan et al. 2001), tobacco (Sweetman et al. 2002; Schaarschmidt et al. 2004), potato (Sweetman et al. 2002; Junker et al. 2003), oilseed rape (Sweetman et al. 2002), and tomato (Garoosi et al. 2005). In plants, alcohol-inducible expression systems responded to treatments by various chemical inducers such as ethanol (Caddick et al. 1998), acetaldehyde (Ac) (Schaarschmidt et al. 2004), and 2-butanone (2-B) (Junker et al. 2003).

Apart from the preliminary observation made by Busov et al. (2005), we know of no published reports of *alc* system being tested in *Populus* species (aspens and cottonwoods). Poplars are not only commercially important, but they are also widely considered to be *the* model tree for genomics and biotechnology (Brunner et al. 2004; Strauss and Martin 2004). The availability of the complete *Populus* genome sequence (JGI, http://genome.jgi-psf/poptr1/poptr1.home.html) adds to its utility as a model system. The aim of this study was to determine whether the *alc* system can function effectively in poplar, and to examine several of its properties. This includes the response of the *alc* system to different chemical inducers, the dose-dependency and time-dependency of induction, different methods of ethanol application, and whether the induction could be observed in potted plants. We will show that the alcohol-inducible system is functional and robust in transgenic *Populus*.

Materials and methods

Plant genotypes, bacterial strains and vectors

Hybrid aspen clones INRA 717-1B4 (female, *Populus tremula* × *P. alba*) and INRA 353-38 (male, *P. tremula* × *P. tremuloides*) were used for all transformations.

Forty- to fifty-day-old, *in vitro* grown poplar plantlets served as explant sources. Micro-cuttings of 717-1B4 and 353-38 were initially cultured on hormone-free, half-strength Murashige and Skoog medium (MS) (Murashige and Skoog 1962). Shoot cultures were maintained on the same medium and grown at 25°C under a 16-h photoperiod [fluorescent tubes (TL70, F25T8/TL735, Philips) at a photon flux density of 45 μE m^{-2} s^{-1}]. The binary vector pJH0143 was provided by Syngenta Biotechnology, Inc. (Research Triangle Park, NC). A diagrammatic representation of the T-DNA from pJH0143 is shown in Fig. 1. *Agrobacterium strain* C58/pMP90 (GV3101), a disarmed derivative of the nopaline strain (Koncz and Schell 1986), was transformed with pJH0143 using the freeze-thaw method of Holstein et al. (1978).

Plant transformation

Agrobacterium cells harboring the binary vector were grown for 24 h in Luria Butani (LB) medium (Weigel and Glazebrook 2002) supplemented with 50 mg/l rifampicin, 50 mg/l kanamycin, and 50 mg/l gentamycin on an orbital shaker at 28°C and 250 rpm. The cells were pelleted by centrifugation at 3,500 rpm (1,992 RCF) for 30–40 min and then resuspended in sufficient *Agrobacterium*-induction medium (IM) (Han et al. 2000) to achieve an OD_{600nm} of 0.5–0.6. Inter-nodal stem segments (3–4 mm in length) and leaf discs (4 mm in diameter) were wounded with multiple fine cuts and incubated in *Agrobacterium* suspension with slow agitation for 1 h. The inoculated explants were cocultivated in callus-induction medium (CIM) [MS supplemented with 10 μM naphthaleneacetic acid (NAA) (Sigma, St. Louis, MO) and 5 μM N^6-(2-isopentenyl)adenine (Sigma)] at 22°C in darkness for 2 days. Explants were then washed four times in sterile, deionized water and once with wash solution (Han et al. 2000). For selection of transformed calli, explants were transferred to CIM containing 50 mg/l kanamycin and 200 mg/l timentin for 21 days. Shoots were induced by culturing explants on SIM medium [MS containing 0.2 μM TDZ (NOR-AM Chemical Co., Wilmington, DE), 100 mg/l kanamycin, and 200 mg/l timentin (GlaxoSmithKline Inc., Research Triangle Park, NC] for 2 to 3 months, subculturing at 3- to 4-week intervals. For shoot elongation, explants were transferred onto MS medium containing 0.1 μM 6-benzylaminopurine (BAP) (Sigma), 100 mg/l kanamycin, and 200 mg/l timentin. The regenerated shoots were rooted on half-strength MS medium supplemented 0.5 μM indole-3-butyric acid (IBA) (Sigma) and 25 mg/l kanamycin. After approximately 30 days, the rooted shoots were micropropagated on the same medium. To ensure that the transformation events are independent, only a single clone per individual explant was selected for further propagation after confirmation of transgene presence by PCR.

All media were autoclaved at 120°C and for 20 min. Vitamins, growth regulators, and antibiotics were filter-sterilized and added to medium after autoclaving. All media were adjusted to a pH of between 5.6 and 5.8 before

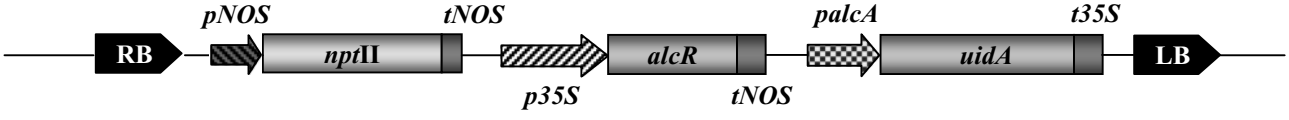

Fig. 1 Schematic representation of T-DNA portion of pJH0143 vector. LB and RB – left and right T-DNA borders, respectively. p*35S* – CaMV *35S* promoter; *alcR* – alcohol sensor protein; p*alcA* – *alcA* promoter of alcohol dehydrogenase from *A. nidulans*; *uidA* – *gus* reporter gene; *t35S* – CaMV *35S* terminator; *pNOS* and *tNOS* – nopaline synthase promoter and terminator, respectively; *nptII* – neomycin phosphotransferase II. Genes are not drawn to scale

autoclaving except LB and IM, which were adjusted to pH 7.0 and 5.0, respectively.

Genomic DNA isolation and PCR amplification

Genomic DNA was isolated from poplar leaves using a Plant DNAeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Approximately 25–50 ng of poplar DNA was used as template for the polymerase chain reaction (PCR). The transgene presence was confirmed by using primers specific for *nptII* (5′-ATCCATCATGGCTGATGCAATGCG-3′ and 5′-CCATGATATTCGGCAAGCAGGCAT-3′) and *uidA* (5′-TGGGCATTCAGTCTG-3′ and 5′-GTGATATCGTCC ACCCA-3′) to amplify 253- and 509-bp products, respectively. A 490-bp fragment encompassing the *palcA*::*uidA* junction was amplified using the following primers: 5′-GTGCTCTCCTACCCCAGGAT-3′ and 5′-TTCACACAAACGGTGATACG-3′.

To amplify the *nptII* junction and *alcA::uidA* junction specific fragments, the conditions used for 30 cycles were: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Conditions for amplification of the *uidA* gene for 30 cycles were: 94°C for 1 min, 61°C for 1 min, and 72°C for 1 min. The PCR products were separated on 1% agarose gels and stained with ethidium bromide.

Induction methods

For induction by a single pulse, we used a combination of the root drench/vapor method. Four milliliters of the inducer solution (ethanol, acetaldehyde, or 2-butanone) were applied directly to the solid medium in a sealed Magenta box. Poplar tissues were harvested for the GUS activity assays 5 days after induction. For continuous, long-term induction, transgenic plants in the Magenta boxes were treated with ethanol vapors for 30 days by dispensing 4 ml of 2% ethanol into a small, open container on the bottom of the Magenta box; the ethanol was changed every 5 days.

In the greenhouse, 200 ml of aqueous solution of the inducer was drenched into the soil (in a pot with an approximate volume of 1.5 l). Pots and 3-month-old poplar plants were then enclosed into sealed light-transparent plastic bags together with separate trays containing 200 ml of the inducer solution for vapor induction. The inducer solution in the tray was changed every 3 days. Soil in pots was drenched with 200 ml of the inducer solution with a 3 days interval during a 10-day induction period.

GUS activity assays

For histochemical GUS staining, tissues were incubated for 6–12 h in 2 mM of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) solution at 37°C essentially as described (Weigel and Glazebrook 2002). After staining, whole plants were treated for 30 min in 10% aqueous solution of commercial bleach (5.25% sodium hypochlorite), transferred to a 70% ethanol, and photographed using an Olympus C5050 digital camera. Individual plant organs were examined and photographed using Zeiss Stemi SV 11 dissection microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

β-Glucuronidase activity *in vitro* was determined in triplicates using fluorometric assay and the GUS substrate 4-methylumbelliferyl β-D-glucuronide (4-MUG) essentially as described at: http://www.markergene.com/product_sheets/pis0877.pdf. GUS activity was calculated as a mean of measurements from leaves (top, middle and bottom) of three individual plants until stated otherwise. The release of 4-methyl umbelliferone (4-MU) was measured using DynaQuant fluorometer (Amersham Biosciences Corp., Sunnyvale, CA) or Wallac Victor^{2}1420 Multilabel counter (Perkin Elmer, Boston, MA) at 15-, 30-, and 75-min time points. To determine the total protein concentration and released 4-MU in the GUS quantification assays, calibration curves were developed using Bradford protein assay (Bradford 1976) and standard 4-MU dilutions, respectively.

Results

Populus transformation

Two genetically distinct *Populus* clones—717-1B4 and 353-38—were used for *Agrobacterium* transformation. The T-DNA of pJH0143 vector (Syngenta Biotechnology, Inc., Research Triangle Park, NC) carries alcohol-inducible switch cassette *35S*::*alcR-alcA::uidA::t35S* similar to described in Sweetman et al. (2002). The expression of AlcR alcohol sensor is driven constitutively by cauliflower mosaic virus (CaMV) *35S* promoter (Franck et al. 1980). The *uidA* reporter gene is transcriptionally fused to *alcA* promoter (Kulmburg et al. 1992) (Fig. 1).

A total of 33 independent kanamycin-resistant lines (transformation events) yielded a PCR product of the expected size for both *nptII* and *uidA* (Table 1). To confirm the integrity of the T-DNA in each line we further used PCR primers bracketing the *alcA*::*uidA* junction. We amplified a product of the expected size (490 bp) from 15 transgenic events in clone 717 and 5 events in clone 353 (approximately 60% of all lines tested).

Alcohol inducibility

A total of 20 selected events were subjected to testing for ethanol induction of GUS activity by using the root drench/vapor combination, as described in the section "Materials and methods". Only some of PCR-positive events showed β-glucuronidase (GUS) activity upon induction with 2% ethanol. Induction was observed in 60% (9 out of 15) of the lines produced in clone 717 and 20% (1 out of 5) of those in 353-38. Ten out of 15 lines confirmed via

Table 1 Transformation frequencies and proportion of ethanol-inducible independent poplar events transformed with pJH4103

Poplar clone	717-1B4	353-38
Total number of explants	1124	704
Explants rooted on kanamycin medium	107	25
PCR positive events		
509 bp (base pairs) *uidA* and 253 bp *nptII* fragments	25	8
490 bp *palcA*::*uidA* fragment	15	5
Number and frequency (%) of lines with alcohol-inducible GUS activity	9 (60)	1 (20)

PCR to contain the intact *alcA*::*uidA* junction also tested positive for the GUS activity (Table 1).

To ensure that there is no basal *alcA* promoter leakage, the clones of all 10 selected lines were mock-induced with water and histochemically stained for GUS. No consistent GUS staining background (except rare occurrences of weak, sporadic spots) was detected in all negative controls (Fig. 2, data not shown) suggesting that the *alc* system is tightly regulated in poplar. Three lines (the clones of events 191, 173 and 183) showing ethanol-inducible GUS staining were selected for further histochemical and fluorometric evaluation of GUS activity.

Distribution of GUS activity in ethanol-induced tissues

Six-week-old poplar plantlets propagated from the primary transformants were treated with chemical inducers using two methods. In the vapor induction method, the inducer was poured into a small open container and then placed into the sealed Magenta boxes. For the root drench/vapor method, sterile inducer solution was poured directly onto the tissue culture medium.

Plants induced by 2% ethanol (v/w) using the root drench/vapor combination showed GUS activity in both leaf and root tissues (Fig. 2). Intense GUS staining was ob-

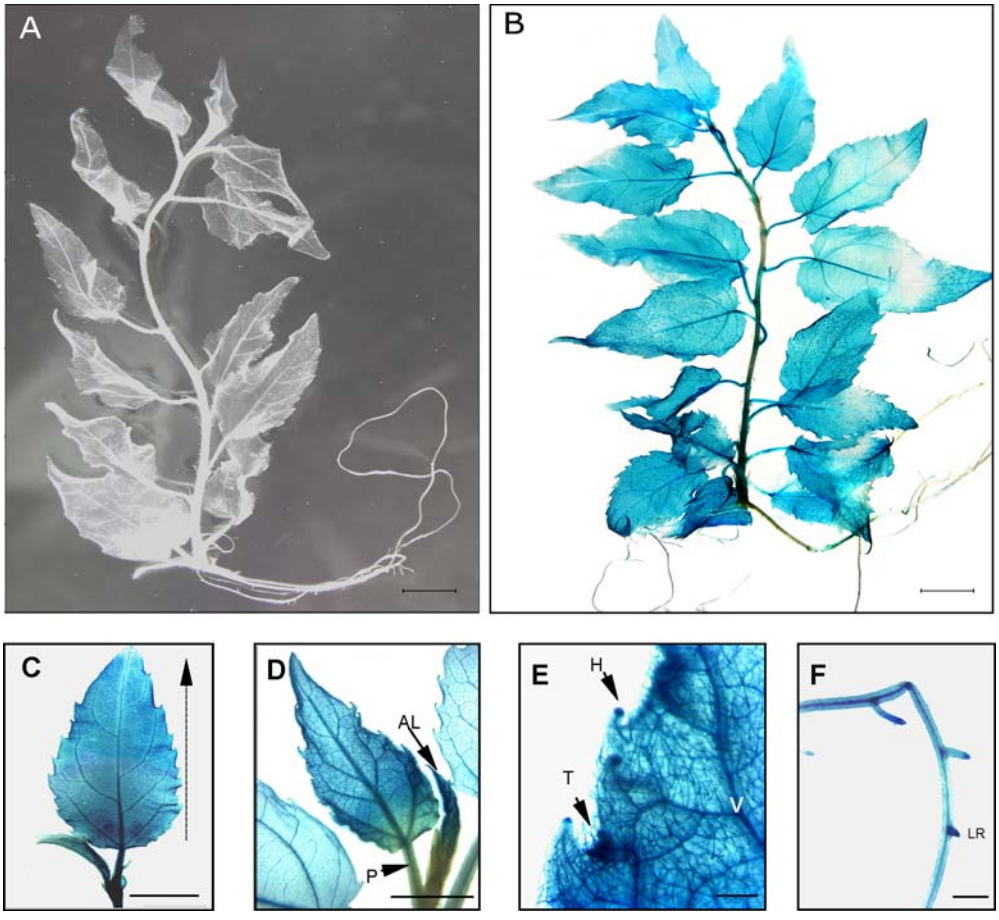

Fig. 2 Histochemical staining for GUS activity in tissues of transgenic *Populus* after induction by ethanol. **a** Control transgenic plantlet stained for GUS activity after mock treatment with water. **b** GUS staining of transgenic poplar induced by 2% ethanol. **c** Young leaf showing a gradient of GUS activity which is stronger in apical part and weaker at the leaf base. Direction of a gradient is indicated by *dashed arrow*. **d** GUS staining of emerging apical leaf (AL). GUS activity is evident only in apical portions of young and emerging leaves. Petiole (P) and leaf base have limited GUS staining. **e** A close-up view of a mature leaf showing intense GUS staining in vascular bundles (V), hydathodes (H), and trichomes (T). **f** A close-up view of an adventitious root. GUS staining is evident in root vasculature and lateral roots (LR). All poplar plantlets (event 191, clone 717) were treated with 2% ethanol using a combination of vapor and root drench methods in enclosed Magenta boxes for 5 days. Scale bars: 1 cm (**a–b**), 0.2 mm (**f**), and 0.5 mm (**e**)

served in leaf vascular bundles, hydathodes, trichomes, and in parenchyma cells. Young and emerging leaves showed a gradient of GUS activity, which was strongest at the apex and weakest at the leaf base. GUS staining was evident in adventitious and lateral roots and to a lesser extent in root hairs. The stems of a majority of induced plants showed weaker GUS activity.

Optimal ethanol concentration and duration of induction period

Clones of three independent transgenic events with high (line 191) and moderate (lines 183 and 173) GUS response were used to determine dose dependency of induction. Plants in the sealed Magenta boxes were treated by various concentrations of ethanol using combination of the root drench and vapor methods for 5 days. Fluorometrically determined GUS activity was detected in the leaves of line 173 at ethanol concentrations as low as 0.5% (v/v) (Fig. 3 and Supplementary Material S1). GUS activity increased with increasing ethanol concentration up to 2% in a dose-dependent manner, but further increases in ethanol concentration led to a reduction in GUS activity. Lines 191 and 183 demonstrated generally similar response except ethanol-induced GUS activity in leaves of line 191 was higher than in lines 173 and 183 (Fig. 3 and S1). Control transgenic plants where ethanol was substituted by an equal volume of sterile water consistently showed negligibly low levels of GUS activity for all the three lines suggesting that *alc* system has a minimal leakage in poplar transgenics.

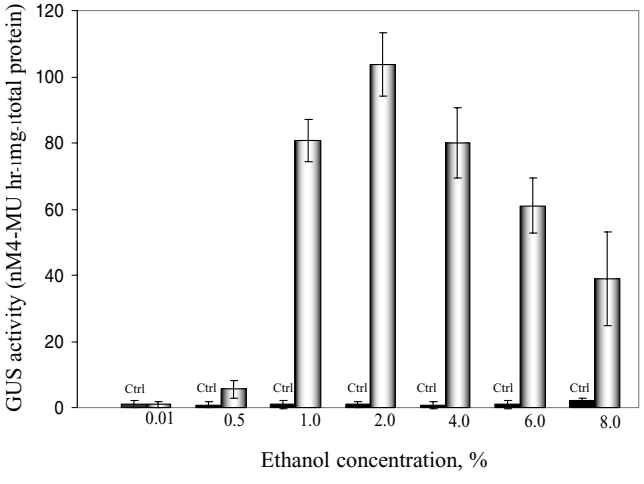

Fig. 3 Dose dependency of ethanol induction. Poplar transgenics (event 173, 717-1B4) were induced with varying concentrations of ethanol for 5 days using combination of vapor and root drench methods in tissue culture conditions. Ctrl – control (transgenic plants mock-induced with water) (shown by filled bars). GUS activity of ethanol-treated plantlets is shown by *open bars*. *Numbers* indicate ethanol concentration. GUS activity was measured in triplicate in three leaves (harvested from lower, middle and top position of a single transgenic plantlet. *Error bars* indicate standard error of the mean

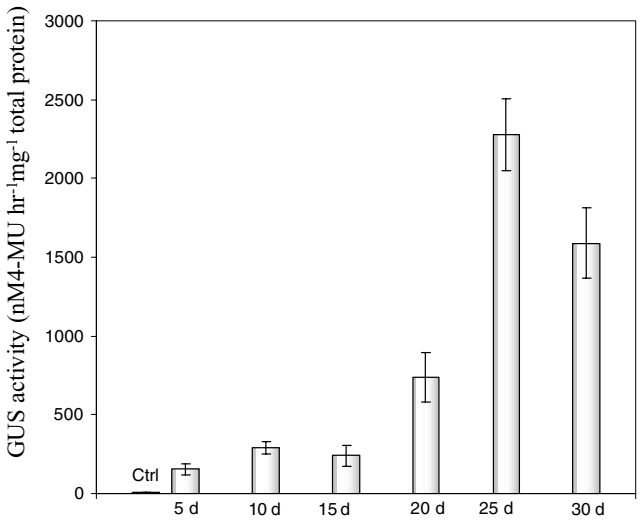

Fig. 4 Time course of continuous induction by ethanol vapor. Transgenic poplar plants (event 191, 717-1B4) were treated continuously for 30 days by ethanol vapor under *in vitro* conditions as described in the section "Materials and methods". GUS activity at each time point (5, 10, 15, 20, 25 and 30 days post-induction) represents a mean of measurements in the leaves harvested from three individual plantlets. *Error bars* indicate standard error of the mean. Ctrl – water-treated control

Continuous exposure to ethanol vapors resulted in a significant increase in induced GUS activity in the leaves of *alcA*::*uidA* plants, for up to 25 days, at which time GUS activity was approximately 10-fold higher than at day 5 (Fig. 4).

Efficiency of various induction agents

The efficiency of *alcA* promoter activation was compared for three chemical inducers: ethanol, Ac, and 2-B (Fig. 5). GUS activity was determined in leaf and root tissues of transgenic poplars induced with 2% ethanol, 0.1% Ac, or 2% 2-B. Ac and 2-B were chosen because both compounds have been shown to activate the *alcA* response system in transgenic potato (Junker et al. 2003). Ac was also reported to be an effective tissue-specific activator of the *alcA* promoter in tobacco (Schaarschmidt et al. 2004). Acetaldehyde was used at a concentration that was 20-fold lower than ethanol because concentrations higher than 0.1% consistently damaged *in vitro*-grown poplar plantlets (data not shown). To achieve the simultaneous induction of both roots and shoots in tissue culture, we used the root drench–vapor combination described in the section "Materials and methods".

Fluorometric determination of GUS activity at 5 days postinduction (dpi) revealed that ethanol was the most efficient inducer of the *alc* system in both leaves and roots, when compared to Ac and 2-B (Fig. 5). GUS expression was lower in roots than leaves but significantly higher than in the control plants. Induction by 2-B had only a marginal effect on GUS activity both in leaves and roots. Induction by 0.1% Ac yielded GUS activity that was 3-fold lower

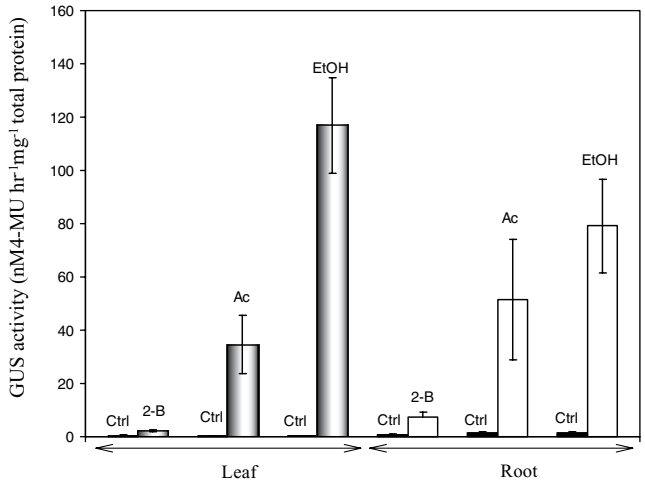

Fig. 5 Response of the *alc* system in *Populus* transgenics to different chemical inducers. GUS activity in the leaf and root tissues of poplar plantlets was induced using 2% 2-butanone (2-B), 0.1 % acetaldehyde (Ac), or 2% ethanol (EtOH). A combination of vapor and root drench methods was used for induction of propagated transgenic clones (event 191) during 5 days in tissue culture. GUS activity at each time point was measured in leaf tissues of three individual plantlets. Ctrl – control. *Error bars* denote standard error of the mean

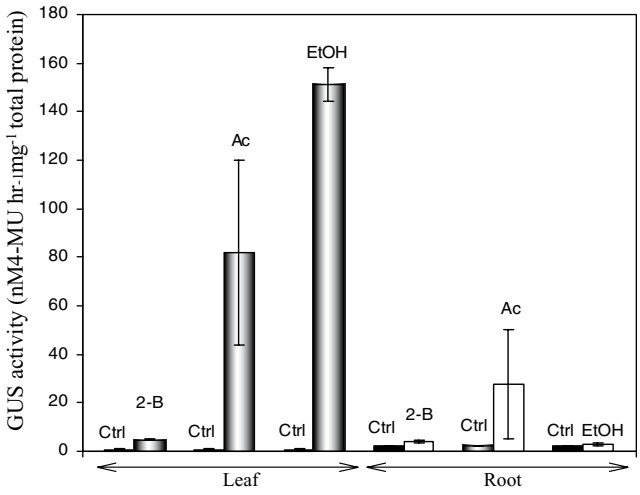

Fig. 6 The *alc* system induction in transgenic poplar under greenhouse conditions. GUS activity in leaf and root tissues of poplar was induced with 2% 2-butanone (2-B), 0.2 % acetaldehyde (Ac), or 2.5% ethanol (EtOH). A combination of vapor and root drench methods was used for 10 days of continuous treatment as described in Materials and Methods. Three-month-old trees in pots with soil were treated by inducers in sealed bags as described in the section "Materials and methods". GUS activity was measured in the leaves and roots of the three cloned plants of transgenic event 191. Ctrl – control. *Error bars* denote standard error of the mean

in leaves, than the response seen for ethanol. GUS levels induced by both ethanol and Ac in roots were significantly higher than in water-treated control, however, the difference between ethanol and Ac induction was marginal. The weaker induction of GUS activity by Ac vapors was consistent with weaker histochemical GUS staining (data not shown).

Induction efficiency under greenhouse conditions

In a pilot study, we simultaneously tested the effect of a root drench and vapors on *alc* induction in a greenhouse environment. For the latter treatment, plants in pots with soil were watered with inducer solution enclosed with an open tray containing the same inducer solution in a plastic bag as described in the section "Materials and methods". Under these conditions, 2.5% ethanol was the most effective inducer after 10 days of treatment. Again, Ac was a weaker inducer at a concentration of 0.2% (Fig. 6). An Ac concentration of 2% was phytotoxic for both the root drench and root drench/vapor methods (data not shown). Induction by 2% (v/v) 2-B had a marginal effect on the GUS expression. No significant GUS activity was detected in the roots of the greenhouse-induced transgenic trees when using root drench method for all the three inductive agents.

When young trees (approximately 3-month-old) were treated continuously for 10 days using the root drench/ vapor combination with aqueous solutions of 2.5% ethanol, 0.2% Ac, and 2% 2-B, there were no obviously detrimental effects on the plant growth and development. Apart from the short-term effects on shape due to confinement in tight plastic bags, both the control and the treated transgenic trees appeared healthy when examined up to 40 days after the cessation of treatment (Fig. 7).

Discussion

We investigated the function of an alcohol-responsive gene induction system in transgenic *Populus*. Using multiple independent transformation events in two different genetic backgrounds we studied the efficiency, optimal concentrations, and induction time. Under the conditions tested, ethanol was the most effective inducer; both Ac and 2-B were weaker inducing agents. Even though the conditions of treatment (i.e., inducer concentration, application method, and induction period) differed for poplar this conclusion was generally consistent with the results seen for potato (Junker et al. 2003). At concentrations higher than 0.1 and 0.2% (v/v), Ac was detrimental to plants both in the tissue culture and in the greenhouse, causing obvious tissue damage and/or necrosis.

Ac was reported to be an effective temporal local inducer of GUS activity in tobacco roots at low concentrations (0.05% or less) (Schaarschmidt et al. 2004), and activated *alc* gene expression in potato tubers more rapidly and at lower concentrations than did ethanol (Junker et al. 2003). Therefore, Ac could be an inducer of choice in applications requiring rapid or localized response such as the expression or suppression of genes regulating the key aspects of poplar development, particularly during regeneration or in localized tissues after infiltration. Further, optimization of Ac delivery to the root or leaf tissues may leads to the

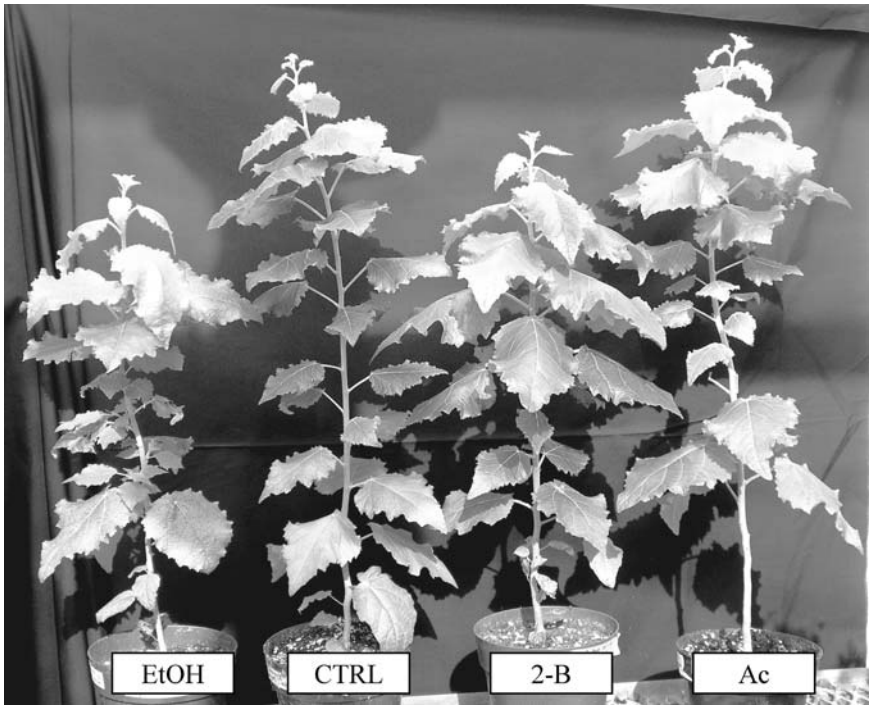

Fig. 7 Recovery of poplar trees after chemical induction under greenhouse conditions. Picture shows the same transgenic trees used for determination of GUS activity in Fig. 6. Trees were grown for additional 40 days after termination of the 10-days induction period. No apparent changes in the plant size or morphology were evident in comparison with water-treated transgenic controls. 2-B – 2% 2-butanone, Ac – 0.2% acetaldehyde, EtOH – 2.5% ethanol, and CTRL – water-treated control

development of local *alc* induction system in poplar similar to that described for tobacco (Schaarschmidt et al. 2004).

In contrast to Ac, ethanol, in concentrations up to 8%, had no apparent effect on the *in vitro* or greenhouse-grown plant. Detectable GUS activity was induced by ethanol in tissue culture at concentrations as low as 0.5%. Optimal concentrations for maximal ethanol induction ranged from 2 to 4%.The basal expression of GUS driven by the *alcA* promoter was minimal in the absence of the inducer, suggesting tight control of the *alc* system in poplar.

Induction appeared to be stronger in plants in tissue culture than in those grown in the greenhouse, possibly because of the limited exposure of cells in mature tissues to the inducer. Inefficient induction of roots was likely the result of low rates of inducer diffusion into the soil. A similar problem was encountered for the developing potato tubers, where the response time of the *alc* system was improved by substituting ethanol with Ac (Junker et al. 2003).

The tissue and organ distribution of the reporter gene activity in various *Populus* tissues suggest that an efficient alcohol induction can be achieved in most of the aerial organs. Unfortunately, little to no induction was observed in stems. This may be a limitation of this system, as *Populus* represents an excellent model system for the study of wood formation. Therefore, a modification of this system may be needed. Xylem-specific promoters have been described (Wu et al. 2000) and tissue-specific alcohol inducible expression has already been demonstrated (Maizel and Weigel 2004; Grandjean et al. 2004).

The suitability of different inducible systems for genomics and other technological applications depends on a number of factors. These include: responsiveness; expression levels in transgenics; basal, uninduced expression; toxicity; and environmental concerns for various inducing chemicals (Padidam 2003). Histochemical staining showed low background GUS activity in the absence of ethanol for the majority of transformation events. Further, the fluorometric GUS quantification in the leaves of three selected independent lines consistently confirmed low basal levels of enzyme in the absence of inducer. The tight regulation of the *alcA* promoter in poplar was consistent with the results reported for a wide range of plant species, including *Arabidopsis* (Roslan et al., 2001), tobacco (Salter et al., 1998), potato (Sweetman et al., 2002; Junker et al., 2003), oilseed rape (Sweetman et al. 2002), and tomato (Garoosi et al. 2005). Our results demonstrate that the alcohol-inducible system is highly efficient in *Populus*; simple, with negligible basal expression; and low toxicity both for the plant, the researcher, and the environment. The latter is particularly important in *Populus*, where large physical size requires testing in greenhouse and field conditions, where confinement of hazardous inducing agents would be problematic.

Chemically inducible gene switches, including this alcohol-inducible system, could be especially valuable for identifying and studying the functionality of candidate genes. Overexpressing or silencing genes regulating key developmental events could be lethal to the modified plants or preclude regeneration altogether. Further modifications of the *alcR-alcA* system by substituting *35S* with the promoter from *LEAFY* allowed for inducible expression of the GUS (Maizel and Weigel 2004) and green fluorescent protein (Grandjean et al. 2004) reporter genes in specific cell types during development of shoot apical meristems of *Arabidopsis*. The *alcR-alcA* system was also used successfully to induce transient gene silencing via RNA interference (Chen et al. 2003; Lo et al. 2005). The *alcR-alcA* appears to be a sensitive and robust inducible system with promise for

applications in functional genomics and aspects of poplar biotechnology.

Acknowledgements We thank Syngenta Biotechnology, Inc. for providing pJH0143, and Dr. Renee Arias for the critical discussions during manuscript preparation. This work was supported in part by grants from the Office of Science (BER), U.S. Department of Energy, grant no. DE-4000023558 "Genome-enabled discovery of carbon sequestration genes in poplar"), the National Science Foundation Industry/University Cooperative Research Centers (award no. 9980423), and by members of the Tree Biosafety and Genomics Research Cooperative based at Oregon State University (http://wwwdata.forestry.oregonstate.edu/tgbb/).

References

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem 72:248–254

Brunner AM, Busov VB, Strauss SH (2004) Poplar genome sequence: functional genomics in an ecologically dominant plant species. Trends Plant Sci 9:49–56

Busov VB, Brunner AM, Meilan R, Filichkin S, Ganio L, Gandhi S, Strauss SH (2005) Genetic transformation: a powerful tool for dissection of adaptive traits in trees. New Phytol 167:9–18

Caddick MX, Greenland AJ, Jepson I, Krause KP, Qu N, Riddell KV, Salter MG, Schuch W, Sonnewald U, Tomsett AB (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat Biotechnol 16:177–180

Chen S, Hofius D, Sonnewald U, Bornke F (2003) Temporal and spatial control of gene silencing in transgenic plants by inducible expression of double-stranded RNA. Plant J 36:731–740

Deveaux Y, Peaucelle A, Roberts GR, Coen E, Simon R, Mizukami Y, Traas J, Murray JAH, Doonan JH, Laufs P (2003) The ethanol switch: a tool for tissue-specific gene induction during plant development. Plant J 36:918–930

Flipphi M, Mathieu M, Cirpus I, Panozzo C, Felenbok B (2001) Regulation of the aldehyde dehydrogenase gene (*alda*) and its role in the control of the coinducer level necessary for induction of the ethanol utilization pathway in *Aspergillus nidulans*. J Biol Chem 276:6950–6958

Franck A, Guilley H, Jonard G, Richards K, Hirth L (1980) Nucleotide sequence of cauliflower mosaic virus DNA. Cell 21:285–294

Garoosi GA, Salter MG, Caddick MX, Tomsett AB (2005) Characterization of the ethanol-inducible *alc* gene expression system in tomato. J Exp Bot 56:1635–1642

Grandjean O, Vernoux T, Laufs P, Belcram K, Mizukami Y, Traas J (2004) *In vivo* analysis of cell division, cell growth, and differentiation at the shoot apical meristem in *Arabidopsis*. Plant Cell 16:74–87

Han K-H, Meilan R, Ma C, Strauss SH (2000) An *Agrobacterium tumefaciens* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*). Plant Cell Rep 19:315–320

Holstein M, De Wacek D, Depicker A, Messers E, van Montagu M, Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens*. Mol Gen Genet 163:181–187

Junker BH, Chu C, Sonnewald U, Willmitzer L, Fernie AR (2003) In plants the *alc* gene expression system responds more rapidly following induction with acetaldehyde than with ethanol. FEBS Lett 535:136–140

Koncz C, Schell J (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204:383–396

Kulmburg P, Judewicz N, Mathieu M, Lenouvel F, Sequeval D, Felenbok B (1992) Specific binding sites for the activator protein, ALCR, in the *alcA* promoter of the ethanol regulon of *Aspergillus nidulans*. J Biol Chem 267:21146–21153

Lo C, Wang N, Lam E (2005) Inducible double-stranded RNA expression activates reversible transcript turnover and stable translational suppression of a target gene in transgenic tobacco. FEBS Lett 579:1498–1502

Maizel A, Weigel D (2004) Temporally and spatially controlled induction of gene expression in *Arabidopsis thaliana*. Plant J 38:164–171

Mathieu M, Fillinger S, Felenbok B (2000) *In vivo* studies of upstream regulatory *cis*-acting elements of the *alcR* gene encoding the transactivator of the ethanol regulon in *Aspergillus nidulans*. Mol Microbiol 36:123–131

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Padidam M (2003) Chemically regulated gene expression in plants. Curr Opin Plant Biol 6:169–177

Roslan HA, Salter MG, Wood CD, White MRH, Croft KP, Robson F, Coupland G, Doonan J, Laufs P, Tomsett AB, Caddick MX (2001) Characterization of the ethanol-inducible alc gene-expression system in *Arabidopsis thaliana*. Plant J 28:225–235

Salter MG, Paine JA, Riddell KV, Jepson I, Greenland AJ, Caddick MX, Tomsett AB (1998) Characterisation of the ethanol-inducible *alc* gene expression system for transgenic plants. Plant J 16:127–132

Schaarschmidt S, Qu N, Strack D, Sonnewald U, Hause B (2004) Local induction of the *alc* gene switch in transgenic tobacco plants by acetaldehyde. Plant Cell Physiol 45:1566–1577

Strauss SH, Martin FM (2004) Poplar genomics comes of age. New Phytol 164:1–4

Sweetman JP, Chu C, Qu N, Greenland AJ, Sonnewald U, Jepson I (2002) Ethanol vapor is an efficient inducer of the *alc* gene expression system in model and crop plant species. Plant Physiol 129:943–948

Weigel D, Glazebrook J (2002) *Arabidopsis*: A laboratory manual. Cold Spring Harbor Laboratory Press,Cold Spring Harbor, NY

Wu L, Joshi CP, Chiang VL (2000) A xylem-specific cellulose synthase gene from aspen (*Populus tremuloides*) is responsive to mechanical stress. Plant J 22:495–502