The *alc-GR* System. A Modified *alc* Gene Switch Designed for Use in Plant Tissue Culture[1][w]

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The ALCR/*alcA* (*alc*) two-component, ethanol-inducible gene expression system provides stringent control of transgene expression in genetically modified plants. ALCR is an ethanol-activated transcription factor that can drive expression from the ALCR-responsive promoter (*alcA*). However, the *alc* system has been shown to have constitutive expression when used in plant callus or cell suspension cultures, possibly resulting from endogenous inducer produced in response to lowered oxygen availability. To widen the use of the *alc* system in plant cell culture conditions, the receptor domain of the rat glucocorticoid receptor (GR) was translationally fused to the C terminus of ALCR to produce ALCR-GR, which forms the basis of a glucocorticoid-inducible system (*alc-GR*). The *alc-GR* switch system was tested in tobacco (*Nicotiana tabacum*) Bright Yellow-2 suspension cells using a constitutively expressed ALCR-GR with four alternative *alcA* promoter-driven reporter genes: β-glucuronidase, endoplasmic reticulum-targeted green fluorescent protein, *haemagglutinin*, and green fluorescent protein-tagged Arabidopsis (*Arabidopsis thaliana*) Arath;CDKA;1 cyclin-dependent kinase. Gene expression was shown to be stringently dependent on the synthetic glucocorticoid dexamethasone and, in cell suspensions, no longer required ethanol for induction. Thus, the *alc-GR* system allows tight control of *alcA*-driven genes in cell culture and complements the conventional ethanol switch used in whole plants.

The *alc* system is a two-component chemically inducible gene expression system, originally developed as a gene switch in *Aspergillus nidulans* (Waring et al., 1989). It consists of the *alcR*-encoded transcription factor (ALCR) that, in response to exogenous ethanol, drives gene expression from the *alcA* target promoter. This system has been used successfully in a wide range of plant species, including Arabidopsis (*Arabidopsis thaliana*), *Brassica napus*, tobacco (*Nicotiana tabacum*), and *Solanum tuberosum* (Salter et al., 1998; Roslan et al., 2001; Sweetman et al., 2002; Junker et al., 2003). The *alc* system has conferred conditional control over reporter, metabolic, and developmental genes (Caddick et al., 1998; Laufs et al., 2003) and can be induced with either ethanol or acetaldehyde (Junker et al., 2003). Furthermore, a range of tissue- and organ-specific *alc* systems have been characterized for use in Arabidopsis (Deveaux et al., 2003; Maizel and Weigel, 2004).

Plant-conditional gene expression systems (for review, see Gatz, 1997; Zuo and Chua, 2000; Padidam, 2003; Wang et al., 2003) are desirable because constitutively overexpressed transgenes, or RNA interference constructs, often display secondary phenotypes that are difficult to interpret or relate to the function of the gene or pathway in question. For example, plants constitutively expressing yeast invertase displayed an extreme stunted growth phenotype (Sonnewald et al., 1991). When expression of yeast invertase was temporally controlled in tobacco, using the *alc* system, it was possible to induce transgene expression and to assay carbon flux at different stages of development (Caddick et al., 1998). Therefore, conditional expression of genes can reveal a greater range of phenotypes.

We have previously used the *alc* system for the conditional restoration of gene function to the *unusual floral organs* (*ufo*) loss-of-function Arabidopsis mutant at different developmental stages (Laufs et al., 2003). *UFO* is required for the specification of organ identity in the second and third whorls and the proper primordium initiation pattern in the inner three whorls. Timed restoration of *UFO* gene function using the gene switch dissected the temporal requirements for *UFO* during floral development, revealing new roles of *UFO* in the outgrowth of petal primordia that previously

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were not apparent in loss-of-function or constitutive gain-of-function backgrounds (Ingram et al., 1995; Lee et al., 1997). Thus, complex phenotypes can be dissected by restoration of gene function with conditional gene expression. Use of the *alc* system as a switch for complementation experiments in plants appears to be generally applicable, as demonstrated by the conditional complementation of the Arabidopsis *leafy-12* mutant (Maizel and Weigel, 2004).

Although the *alc* system has found widespread utility as a safe and effective expression system in whole plants, there are concerns about its efficacy under plant cell culture conditions. As will be demonstrated here, cell cultures often have significant, and sometimes high, levels of transgene expression in the absence of exogenous inducer. Although the reason for this is unclear, it is likely that plant cell culture conditions result in oxygen limitation leading to the production of inductive compounds. This limits the use of the *alc* system since it is often advantageous to test the effects of transgene expression in cell cultures. Furthermore, in those species in which transformation is reliant upon callus, transformation may be limited if expression of the transgene has deleterious effects during subculture or regeneration. To restore the requirement for an exogenously added chemical inducer, the rat glucocorticoid receptor (GR) domain has been fused to the ALCR transcription factor; conferring steroid-inducible control over *alc*-mediated gene expression. In animals, proteins containing a GR domain are sequestered outside of the nucleus with heat shock proteins and are released from these complexes upon binding of a steroid ligand (Picard, 1994). The rat GR domain, tagged to constitutively expressed transcription factors, has previously been used in plants to confer dexamethasone (Dex)-dependent control over transcription factor function (Sablowski and Meyerowitz, 1998).

In this article, we describe the leaky expression of *alc* in tomato (*Lycopersicon esculentum* L. var. Ailsa Craig Mill.) cell cultures derived from a characterized transgenic line and the specification of a Dex-inducible *alc* system (*alc-GR* system) for use in the tobacco Bright Yellow (BY)-2 cell culture (Nagata et al., 1992). We report that neither addition of ethanol or accumulation of endogenous inductive compounds activates the *alc-GR* system. Moreover, gene expression in the absence of inducer or on addition of ethanol is low or not detectable and responds quickly in a dose-dependant manner to Dex. Therefore, this system presents a useful gene switch for plant cell cultures, which is directly comparable and uses many of the same components as the *alc* system in whole plants.

RESULTS

The *alc* Gene Expression System Is Constitutively Active in Plant Cell Cultures

To test the efficacy of the *alc* system to regulate transgene expression, callus and cell suspension cultures were derived from previously characterized transgenic tomato plants (Garoosi, 1998; G.A. Garoosi, M.G. Salter, M.X. Caddick, and A.B. Tomsett, unpublished data). The highly inducible transgenic line (LeGUS20), carrying *35S*::*ALCR*/*alcA*::*GUS* (Caddick et al., 1998; Roslan et al., 2001), had been selfed, and segregation was consistent with a 3:1 ratio indicating the presence of a single T-DNA insert (data not shown). Seedlings of the line grown in hydroponic solution consistently demonstrated tight regulation of *alc*-directed β-glucuronidase (GUS) expression, having a negligible background activity in the absence of inducer and a high GUS activity 96 h after induction with 0.1% (v/v) ethanol (Fig. 1a). Assays for the presence of alcohol dehydrogenase (ADH) activity in such lines demonstrated a low level of activity comparable with wild-type tomato seedlings grown under identical conditions. Under hypoxic/anoxic conditions, seedlings exhibited significant GUS activity whether ethanol was present or absent in the growth medium (Fig. 1b), and levels of ADH activity consistent with a switch to ethanolic fermentation, in which pyruvate is converted to acetaldehyde by pyruvate decarboxylase and the acetaldehyde is reduced to ethanol by ADH (Drew, 1997; Fukao and Bailey-Serres, 2004). As recently demonstrated, the *alc* system can be induced with either ethanol or acetaldehyde (Junker et al., 2003). Callus and cell suspension cultures derived from the same plant line were also tested for GUS and ADH activity. Such cells demonstrated significant levels of GUS activity in the absence of exogenously applied ethanol and ADH levels indicative of ethanolic fermentation (Fig. 1c). Similar data were obtained with tissue cultures derived from a second tightly regulated transgenic line in which chloramphenicol acetyltransferase was used as the reporter gene for the *alc* system. These cell suspensions were grown in both baffled (to increase aeration) and unbaffled conical flasks, but while expression from the *alcA* promoter declined with increased aeration, a significant amount of uninduced activity was retained (Supplemental Table I).

Expression from the *alc* system in the absence of exogenously supplied inducer is not restricted to tomato. Tobacco BY-2 cells were transformed with *35S*::*ALCR*/*alcA*::*GUS* using the pSRN/AGS plasmid (Roslan et al., 2001). A cell line derived from multiple transformant calli was treated overnight with various concentrations of ethanol and stained for GUS expression (Jefferson et al., 1987). It was clear that GUS expression occurred with or without treatment with ethanol (Fig. 1d). Cells treated with 0% to 1% (v/v) ethanol displayed distinct GUS staining, but 3% (v/v) ethanol was clearly toxic to cells as evidenced by weak GUS staining and distorted cell morphologies. Constitutive expression has also been observed in whole Arabidopsis plants when grown on agar or Phytagel-containing media (data not shown; Roslan et al., 2001).

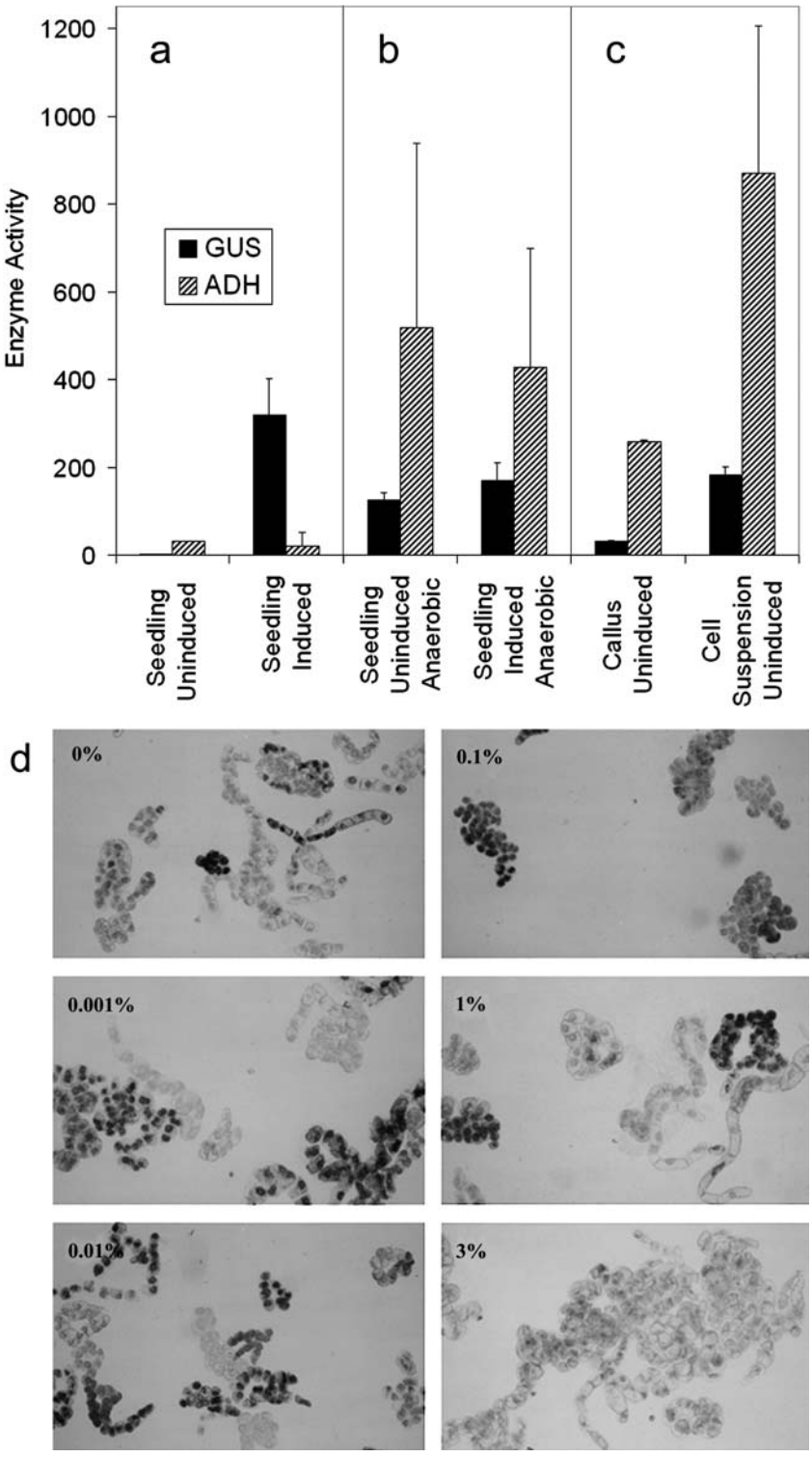

Figure 1. *alc* expression in uninduced cell cultures. Enzyme Activity represents GUS (nmol 4MU h^{-1} mg^{-1} total protein) and ADH (μmol min^{-1} mg^{-1} fresh weight of tissue); the error bars represent SE from at least four replicates for each value. a, Seedlings (20 d old) of LeGUS20, carrying cauliflower mosaic virus *35S*::*ALCR*/*alcA*::*GUS*, were incubated in hydroponic solution without/with 0.1% (v/v) ethanol (Uninduced/Induced) for 96 h, before enzyme extraction to assay for GUS and ADH activity. b, Seedlings of LeGUS20 were incubated in hydroponic solution, purged with 100% N_2 gas to decrease oxygen in the liquid medium, without/with 0.1% (v/v) ethanol (Uninduced/Induced), and sealed in a 3-L plastic box containing Anaerocult (Merck, Rahway, NJ) to induce anoxia for 96 h, before enzyme extraction. c, LeGUS20-derived callus was grown on solid Murashige and Skoog medium (without ethanol) in parafilm-sealed petri dishes for 20 d. Individual calli, 1.5 cm in diameter, were used for enzyme extraction. Cell suspension cultures (originally prepared from LeGUS20 callus) were grown for 2 weeks in the absence of ethanol in 50 mL of liquid medium in a 250-mL conical flask on an orbital shaker at 120 rpm, before enzyme extraction. d, Tobacco BY-2 cells transgenic for *35S*::*ALCR/alcA*::*GUS* were induced with 0%, 0.001%, 0.01%, 0.1%, 1%, and 3% (v/v) ethanol overnight, stained for GUS activity with GUS-staining buffer, and visualized by light microscopy. GUS stained cells appear black.

Modification of the *alc* System and Selection of Transgenic Lines

To confer inducible transgene expression to the *alc* system in BY-2 cells, a translational fusion was constructed that placed the rat GR domain gene fragment (*GR*) at the 3′-end of the *ALCR* gene. The *ALCR-GR* gene fusion was placed under the control of a *35S* promoter in the pGreen 0129 plasmid (Fig. 2a; Hellens et al., 2000) and transformed into BY-2 cells. Eleven independently transformed *35S*::*ALCR-GR* lines (calli/clones) were generated, all of which expressed *ALCR-GR* when assayed by semiquantitative reverse

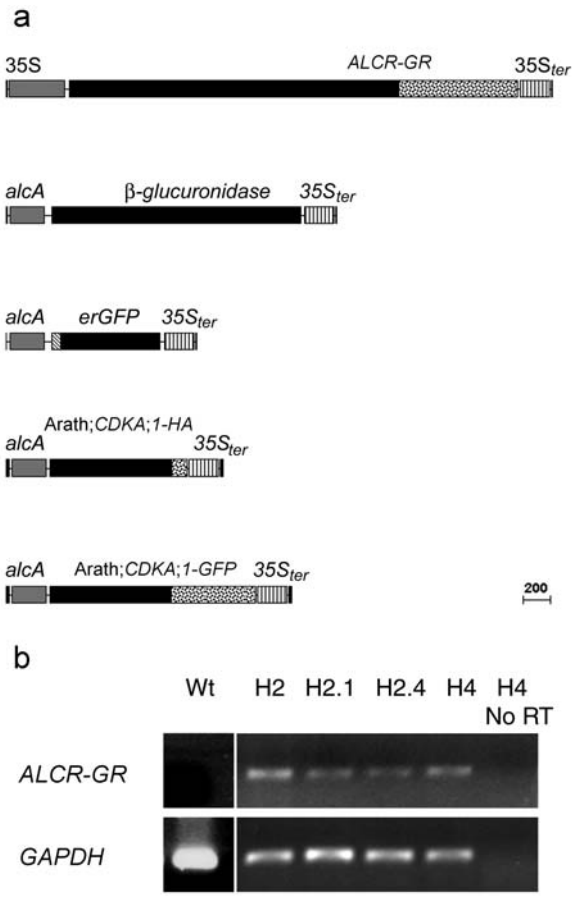

Figure 2. Schematic representation of the constructs used and expression of *ALCR-GR* in primary *alc-GR* system transformants. a, *35S*::*ALCR-GR* in pGreen 0129, *alcA*::*GUS* in pGreen 0029, *alcA*::*erGFP* in pGreen 0029, *alcA*::Arath;*CDKA;1-HA* in pGreen 0029, and *alcA*::Arath;*CDKA;1-GFP* in pGreen 0029. b, *ALCR-GR* and the tobacco control *glyceraldehyde-3-P-dehydrogenase* (*GAPDH*) transcripts were assayed by RT-PCR in the H2, H2.1, H2.4, and H4 independent *35S*::*ALCR-GR* transformants. *GAPDH* transcript, but not *ALCR-GR* transcript, accumulated in untransformed (Wt) BY-2 cells and transcripts for neither are PCR amplified from RNA extracted from a *35S*::*ALCR-GR* line without reverse-transcriptase treatment (H4 No RT). All four *35S*::*ALCR-GR* transformants express both *GAPDH* and *ALCR-GR* transcripts.

transcription (RT)-PCR (Fig. 2b; data not shown). The H2, H2.1, H2.4, and H4 ALCR-GR background lines were chosen for further analysis as they were the first to become established in liquid culture. The ALCR-GR background lines were sequentially transformed with *alcA*::*GUS*, and the resulting double transformants were assayed for Dex-dependent expression by staining for GUS activity. After induction, the proportion of *alcA*::*GUS* sequentially transformed lines expressing GUS varied between the background lines. In the H2 background, five out of 12 were GUS positive, while nine out of 12 H2.1, four out of 12 H2.4, and 10 out of 12 of H4 secondary transformants also displayed GUS staining. Of the GUS positive lines a variable proportion in each background expressed GUS constitutively: three out of five of H2, one out of nine of H2.1, zero out of 12 of H2.4, and one out of 10 of H4, respectively (data not shown). We concluded from this that a screen for conditionally expressing *ALCR-GR* secondary transformants is appropriate prior to experimentation.

The ALCR-GR Transactivator-Mediated Gene Switch Is Dex But Not Ethanol Dependent in BY-2 Cells and Responds to Dex in a Dose-Dependent Manner

To test how the addition of the GR domain to the ALCR protein had altered its response to induction, ALCR-GR control over *alcA*::*erGFP* expression was assayed following treatment of BY-2 cells with different combinations of Dex and/or solvents, in particular ethanol. We visually assayed the expression of endoplasmic reticulum-targeted green fluorescent protein (*erGFP*), in an H2.4 ALCR-GR background line sequentially transformed with *alcA*::*erGFP*, by fluorescence microscopy, 24 h after treatment with 1 μM Dex dissolved in ethanol or dimethyl sulfoxide (DMSO). Both solvents were applied at a final concentration of 0.1% (v/v). The solvents were also applied independently to determine whether they alone could induce expression via ALCR-GR. *erGFP* expression was induced by Dex dissolved in ethanol and by Dex dissolved in DMSO (Fig. 3a). Dex was essential for the activation of the *alc-GR* system as neither solvent alone was able to induce reporter gene expression.

To assess if ALCR-GR-regulated gene expression was dependent on the amount of Dex applied, the levels of *alcA*-driven GUS or Arath;*CDKA;1-haemagglutinin* (*HA*) were measured following treatment of cells with various concentrations of the inducer. GUS enzyme activity was assayed using a fluorometric assay following a 24-h induction with Dex (Fig. 3b). Generally, GUS activity was highest in a range of 0.1 to 10 μM Dex, with a slightly lower activity at 0.005 μM Dex. All three GUS reporter lines displayed a significantly lower GUS activity with 0.001 μM Dex. Two of the three GUS reporter lines displayed no background GUS activity on treatment with DMSO alone; however, line H4 32.8 displayed low GUS activity in the absence of Dex.

The Dex dose-dependent Arath;CDKA;1-HA expression profile was assayed by western blot using whole-cell extracts (Fig. 3c). Arath;CDKA;1-HA expression produces a protein that is 6 kD larger than the endogenous tobacco CDKA protein: both proteins are recognized by the monoclonal antibody raised against the cyclin-dependent kinase (CDK) PSTAIR motif. The *alcA*::Arath;*CDKA;1-HA* in an H2 background displayed a similar Dex dose-dependent Arath;*CDKA;1-HA* expression profile as the GUS lines. Western blots of the Dex dose-dependent erGFP expression also displayed the same induction profile (Fig. 3d).

Time Course of Induction

The dynamics of Dex induction of the *ALC-GR* system in tobacco BY-2 cells were defined by western

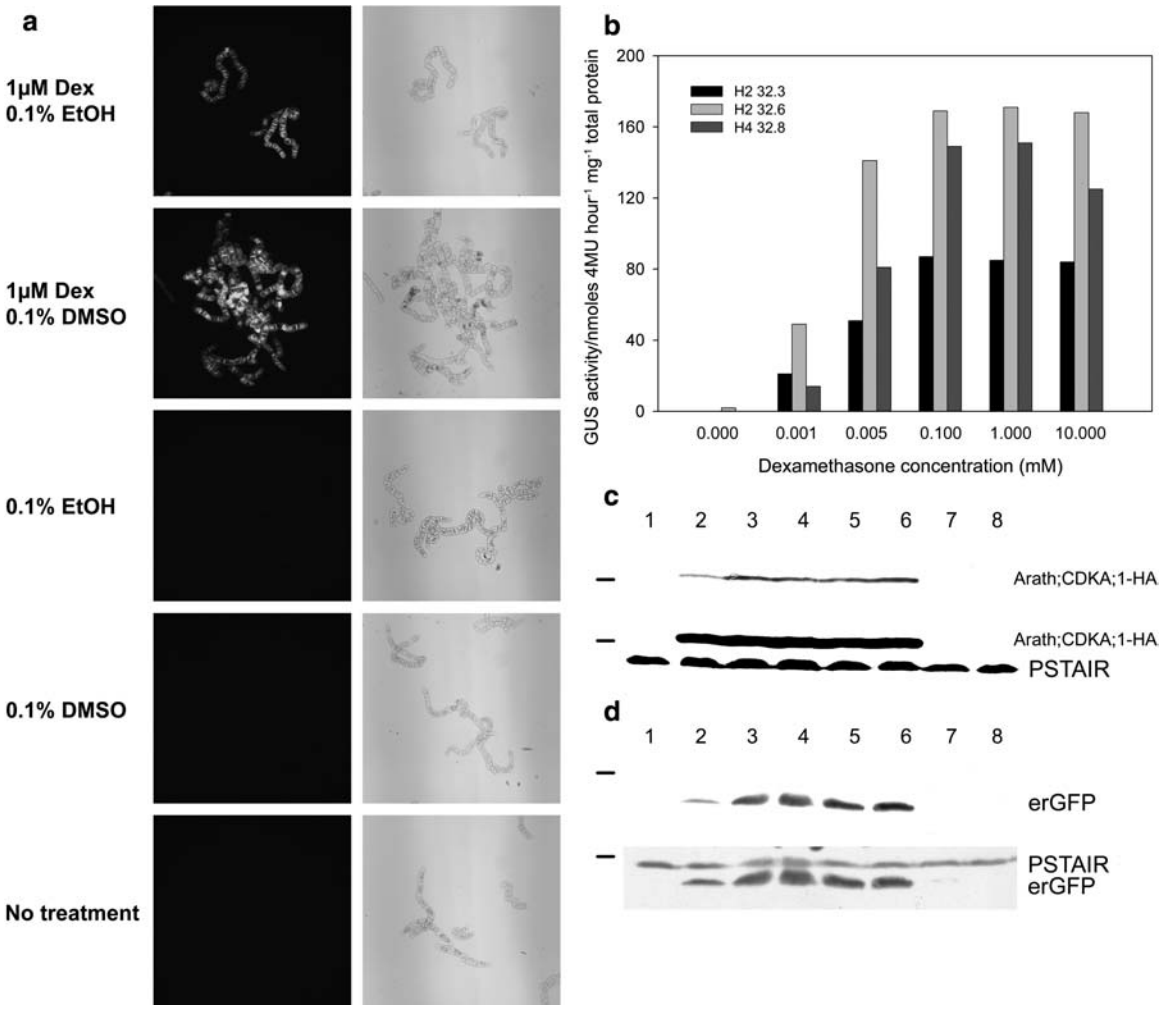

Figure 3. Dex induces the *alc-GR* system in a dose-dependent manner. a, BY-2 cells, transgenic for both *35S*::*ALCR-GR* and *alcA*::*erGFP*, were treated with Dex dissolved in either 0.1% (v/v) ethanol or DMSO, or either solvent without Dex, or finally without solvent or Dex. The erGFP expression was detected by fluorescence microscopy (left-hand column, GFP fluorescence appears white), and the cells are presented as brightfield images (right-hand column). b and c, One day after 1:10 subculture, ALCR-GR background lines and lines secondarily transformed with *alcA* driven reporter constructs were treated with 0 to 10 μM Dex. Twenty-four hours after inductions were applied, one sample of each line was harvested for transgene expression assays. b, Lines H2 32.3, H2 32.6, and H4 32.8, which are H2 and H4 ALCR-GR backgrounds secondarily transformed with *alcA*::*GUS*. The values represent GUS activity (nmol $4MU^{-1}$ mg^{-1} total protein). c, Western blot using PSTAIR antibody to detect both Arath;CDKA;1-HA and endogenous tobacco CDK (PSTAIR) in protein extracts from an H2 ALCR-GR background secondarily transformed with *alcA*::Arath;*CDKA;1-HA*. Lanes 1 to 6 contain protein extracts from an H2-derived BY-2 cell line carrying *35S*::*ALCR-GR/alcA*::Arath;*CDKA;1-HA*, and lanes 7 to 8 contain protein extracts from the H2 ALCR-GR background line. Upper blot represents a shorter exposure time than the lower blot where endogenous CDK (PSTAIR) is also visible. d, Western blot first probed with GFP antibody (top) and later probed with PSTAIR antibody to detect erGFP and tobacco CDK, respectively, in protein extracts from an H2 ALCR-GR background line secondarily transformed with *alcA*::*erGFP*. Lanes 1 to 6 contain protein extract from an H2-derived BY-2 line carrying *35S*::*ALCR-GR/alcA*::*erGFP*, and lanes 7 to 8 contain protein extracts from the H2 ALCR-GR background line. Lane 1, 0 μM; lane 2, 0.001 μM; lane 3, 0.005 μM; lane 4, 0.1 μM; lane 5, 1 μM; lane 6, 10 μM; lane 7, 0 μM; and lane 8, 10 μM Dex. Molecular weight marker on the western-blot figure represents 36 kD.

blotting and fluorescence microscopy analyses of tagged Arath;CDKA;1. Following induction with 0.1 μM Dex, the accumulation of Arath;CDKA;1-HA, driven by the *alc-GR* system, was monitored by western blot using the PSTAIR antibody on whole-cell extracts. The Arath;CDKA;1-HA protein was detectable 1 h after induction, exceeded the level of endogenous CDKA proteins after 2 h, and maintained this high level for at least 12 h (Fig. 4a).

Arath;CDKA;1-GFP, a GFP-tagged Arabidopsis CDK driven by the *alc-GR* system, was visually monitored by fluorescent microscopy, again following treatment with 0.1 μM Dex. The Arath;CDKA;1-GFP fluorescence was weakly fluorescent 4 h after induc-

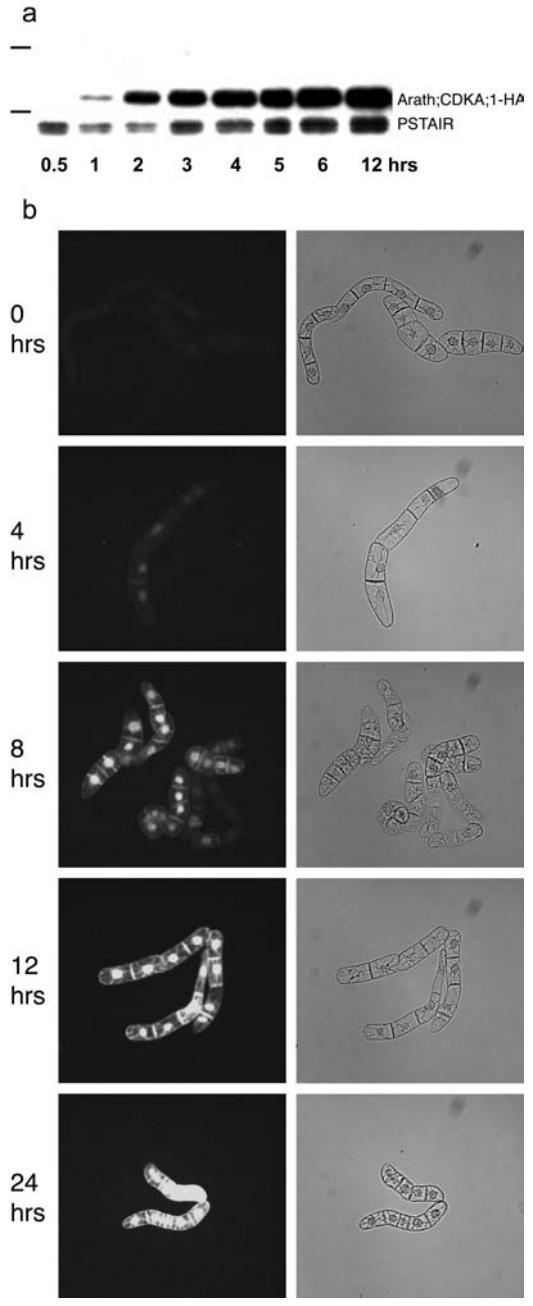

Figure 4. Time course of Dex induction. a, Western blot using PSTAIR antibody to detect Arath;CDKA;1-HA and endogenous tobacco CDK (PSTAIR) in protein extracts from an H2 ALCR-GR background secondarily transformed with *alcA*::Arath;*CDKA*;*1-HA* 0.5 to 12 h following induction with 0.1 μM Dex. Prior to induction, this line had been subcultured by dilution 1:10 from stationary phase cultures and grown for a further 24 h. Molecular weight markers represent 36 and 50 kD. b, Fluorescent microscopy of Arath;CDKA;1-GFP fluorescence in cells of an H2 ALCR-GR background line secondarily transformed with *alcA*::Arath;*CDKA*;*1-GFP*, following treatment as in a. Left-hand column displays Arath;CDKA;1-GFP fluorescence (appears white) and right-hand brightfield view of cells.

tion and was strongest from 12 h onwards after induction (Fig. 4b). It is known that GFP takes up to 4 h to fold properly before it becomes active (Heim et al., 1994). Therefore, it is consistent that the Arath;CDKA;1-GFP fluorescence increases much slower than the Arath;CDKA;1-HA protein accumulates, following Dex induction.

DISCUSSION

Given the increasing use of the *alc* system (Ait-ali et al., 2003; Chen et al., 2003; Deveaux et al., 2003; Maizel and Weigel, 2004), there is a need to widen its applicability to plant culture growth conditions. In this article, we report the leaky expression of the *alc* system in plant tissue cultures and an adaptation of it for use in plant cell suspension cultures, in particular in tobacco BY-2 cells.

The *alc* gene switch has great potential for research and commercial application in part due to the nature of the inducer, ethanol, which is cheap and relatively nontoxic to plants and the environment. Ethanol is also highly penetrating and can induce gene expression deep within tissues such as meristems and even developing seeds (G.R. Roberts, L. Sakvarelidze, P. Laufs, and J.H. Doonan, unpublished data). However, plants can produce ethanol and acetaldehyde (the physiological inducer of the *alc* switch) during ethanolic fermentation, triggered by oxygen deficiency (see Drew, 1997). Typically, in whole plants, this can arise through excess water in the root environment of dryland species. It has previously been reported that the *alc* system is activated by endogenous inducer in experiments designed to lower oxygen levels and when Arabidopsis was grown in agar plates, but this has not been observed for seedlings grown in hydroponic solution or for plants in soil flooded for 3 d (Salter et al., 1998; Roslan et al., 2001). It was possible however that plant cell suspension cultures would also experience oxygen limitation because of their submerged environment and hence induce *alc* in the absence of exogenously applied inducer. If affected, this would severely limit the widespread use of *alc* for those applications in which such cells are the material of choice, such as detailed cell biological studies (Nagata et al., 1992) or the production of bioactive molecules (James and Lee, 2001).

To address this, callus and cell suspension cultures were produced from transgenic plants already shown to contain a single T-DNA, which segregated normally, and that showed inducible regulation by *alc* but with negligible basal activity in the absence of exogenous inducer. This eliminated the possibility of constitutive expression in the cell cultures arising from the position of T-DNA integration, and further avoided the possibility of constitutive expression due to a series of different, or multiple, integration events within the selected transformed callus/cell line. Significant basal expression from *alc* was observed in callus cells, and

high activity for both the *alc*-directed reporter gene activity and ADH were detected without exogenous inducer. The levels of activity in the cell suspension cultures were consistent with the artificial environment used to induce severe hypoxia in seedlings. Attempts to increase oxygen availability to such cells through the use of baffled flasks lowered but did not eliminate the leaky activity. This strongly suggested that ethanolic fermentation is activated in cell suspension cultures.

Since tobacco BY-2 cells are used widely, these were selected to test a modified *alc* system to ensure tight regulation of the transgene. Tests of the unmodified *alc* system showed expression in these cells in the absence of exogenous inducer, as in the tomato lines. We conferred chemical-inducible control over the *alc* system, in culture, by creating a translational fusion between the ALCR transcription factor and the GR domain of the GR to create the *alc-GR* Dex-inducible switch. A detailed characterization of the *alc-GR* switch in BY-2 cells demonstrated that this system mediates both tightly regulated and rapidly induced gene expression. The *alc-GR* system was activated by Dex whether dissolved in ethanol or DMSO, and it was tightly regulated as neither addition of ethanol or DMSO mediated gene expression in the absence of Dex. The switch was dose dependent with respect to Dex in a similar manner to other reports of glucocorticoid-inducible expression in BY-2 cells (Nara et al., 2000). Reporter protein was evident on western blots after only 1 h of induction and highly induced from 2 h onwards. GFP-tagged reporter fluorescence was visible at 4 h, which is consistent with the time it takes GFP to mature and fluoresce (Heim et al., 1994).

CONCLUSION

A modified *alc* switch (*alc-GR*) has been developed and characterized for use in cell culture that complements and extends the existing *alc* switch as used in whole plant systems. Although this has not yet been tested in whole plants, the modified switch should be resistant to endogenous activation by flooding and other stress conditions. Furthermore, constructs containing genes driven by the *alcA* promoter can now be conditionally expressed in plants by induction with ethanol (via ALCR) and in cell culture by induction with Dex (via ALCR-GR). The *alc-GR* system restores conditional control over gene expression for the *alc* system, enhancing the general utility of the *alc* gene switch.

MATERIALS AND METHODS

Plasmid Construction

DNA manipulations and cloning were carried out using standard procedures (Sambrook et al., 1989). The binary vector pSRN::pAGS expressed the *alcR* gene from the cauliflower mosaic virus 35S promoter and the GUS reporter gene from the *alcA* promoter, as described previously (Caddick et al., 1998; Roslan et al., 2001). The *35S::alcR-GR* construct was introduced into the binary transformation vector pGreen0129 (Hellens et al., 2000). For this, a *Kpn*I/*Eco*RV cassette from pJIT60 (Guerineau and Mullineaux, 1993), containing 2 × 35S promoter-35S terminator, was inserted into pGreen0129, generating pGR-2 × 35S. An *Xba*I (end polished with Klenow)/*Bam*HI cassette from pDltaGRBX (gift from Dr. Robert Sablowski), containing *GR*, was inserted into pGR-2 × 35S using *Bam*HI/*Sma*I, generating pGR-2 × 35S-GR. Finally, *Bcl*I and *Sal*I restrictions sites were added to the *alcR* gene by PCR using AATGATCAAGAAAGCTGTCAACTTTCCCATTCAAACC and CCGTCGACGATATTCTCCTGCACACAGCATGG as primers and pSRN1 as template (Caddick et al., 1998). After *Bcl*I and *Sal*I digestion, ALCR was inserted into pGR-2 × 35S-GR, generating pGR-35S::alcR-GR.

The *alcA::GUS-35S terminator* cassette was introduced into the pGreen0029 vector (Hellens et al., 2000) as a *Hin*dIII fragment from pSRN1 AGS/Bin19 (Roslan et al., 2001), generating pGR32. The *Hin*dIII *alcA::erGFP-35S terminator* cassette from pMCB56 (Fernandez-Abalos et al., 1998) was introduced into pGreen0029, again by *Hin*dIII digestion/ligation, generating pGR33.

The *alcA*::Arath;*CDKA;1-HA* cassette was introduced into pGreen0029. For this the *Eco*RI restriction site was added to a triple *HA* tag by PCR using primer set GCGGTAAATCTAGCAGTGCCTCAT and TAGCGAATTCACTGAGCAGCGTAATCTGGAA and pUC-HA as a template (gift from Dr. Laci Bogre), and *Pst*I/*Not*I restriction sites were added to *CDKA;1* by PCR using the primer set AAACTGCAGATGGATCAGTACGAGAAAGTTGAG (*CDKA;1-Pst*I) and AAAGCGGCCGCCAGGCATGCCTCCAAGATCCTTG (*CDKA;1-Not*I) and pRS97 as template (gift from Dr. Robert Sablowski). The *HA* PCR product was digested with *Not*I/*Eco*RI and the Arath;*CDKA;1* PCR product was digested with *Pst*I/*Not*I, and both fragments were introduced into pL4 (gift from Syngenta, Norwich, UK), generating pGR40. A *Hin*dIII partially digested cassette from pGR40, containing *alcA*::Arath;*CDKA;1-HA-35S terminator*, was inserted into pGreen0029, generating pGR42 (Supplemental Fig. 1).

The *alcA*:: Arath;*CDKA;1-GFP* cassette was introduced into pGreen0029. For this, *Not*I/*Eco*RI restriction sites were added to a *GFP* tag by PCR using primer set CAGGGCGGCCGCGGGAGTAAAGGAGAAGAA and CTCGAATTCTTTATTTGTATAGTTCATCCATCGCA and pMCB5 as a template (Fernandez-Abalos et al., 1998), and *Pst*I/*Not*I restriction sites were added to *CDKA;1* by PCR using the *CDKA;1-Pst*I and *CDKA;1-Not*I primer set as for amplification using pRS97 as template. The *GFP* PCR product was digested with *Not*I/*Eco*RI and the Arath;*CDKA;1* PCR product was digested with *Pst*I/*Not*I, and both fragments were introduced into pL4, generating pGR6. A *Hin*dIII partially digested cassette from pGR11, containing *alcA*::*CDKA;1-GFP -35S terminator*, was inserted into pGreen0029, generating pGR14 (Supplemental Fig. 1).

Plant Material, Growth Conditions, Transformation, and Cell Culture Synchronization

The LeGUS20 transgenic tomato (*Lycopersicon esculentum* L. var. Ailsa Craig Mill.) line, transformed with pSRN::pAGS::kan^R, has been described previously (Garoosi, 1998; G.A. Garoosi, M.G. Salter, M.X. Caddick, and A.B. Tomsett, unpublished data). For induction experiments, soil-grown seedlings (16 h light 300 μmol m^{-2} s^{-1}, 25°C ± 2°C and 8 h dark, 16°C ± 2°C) were carefully removed, rinsed, and placed in 120 mL of 0.05% (w/v) Miracle-Gro (Marysville, OH) containing the required concentration of ethanol in a 400-mL Magenta pot. Callus was established from explants of young leaves of LeGUS20 on Murashige and Skoog medium (Murashige and Skoog, 1962), containing 0.8% agar, 2,4-dichlorophenoxyacetic acid (2 μg mL^{-1}), 6-benzylaminopurine (0.5 μg mL^{-1}), and kanamycin (80 μg mL^{-1}) at 26°C under low light (150 μmol m^{-2} s^{-1}) for 16 h light and an 8-h dark photoperiod, and subcultured at intervals of 2 to 3 weeks. Tomato cell suspension cultures were initiated from callus, as described by Patil et al. (1994), in the same medium (as callus) but lacking agar and kanamycin. It was maintained by dilution (1:5) every 2 weeks and incubated at 25°C and with shaking at 120 rpm in the dark. Note that ethanol was not used as a solvent for any components of the tissue culture media.

A rapidly growing suspension culture of tobacco BY-2 cells (*Nicotiana tabacum* cv BY-2) was maintained by weekly dilution (1:100) of culture into fresh medium (Nagata et al., 1992) and cultured at 27°C and 130 rpm in the dark. The pGreen plasmids described above were transformed into the *Agrobacterium tumefaciens* strain GV3101, which had previously been transformed with pSoup (Hellens et al., 2000) by electroporation.

Following cocultivation of BY-2 with pGR-35S::alcR-GR harboring *A. tumefaciens*, stable transformants of BY-2 were selected on petri plates containing 0.4% Phytagel in fresh culture medium, supplemented with carbenicillin (500 μg/mL) and hygromycin (41.6 μg/mL). The resultant ALCR-GR trans-

formants were sequentially transformed with pGR32, pGR33, pGR14, or pGR42, and secondary transformants were selected for on media containing plates supplemented with kanamycin (200 μg/mL; An, 1987). After 3 to 4 weeks, transgenic calli were recovered and replated on fresh Phytagel petri plates, as above. Individual calli were then cultured in liquid medium with antibiotics. Carbenicillin was used for three rounds of subculturing.

Chemicals and Induction

To induce the unmodified *alc* system, the appropriate concentration of ethanol (v/v) was added to the growth medium. Induction of the *alc-GR* system was achieved using Dex (Sigma-Aldrich, St. Louis), dissolved in either ethanol or DMSO. For selecting *alc-GR* lines with high inducible expression, portions of calli/clones were induced overnight in microfuge tubes or 96-well microtitre plates with 500 μL of fresh media containing Dex/DMSO or DMSO and assayed for gene expression as appropriate. Cultures were induced by addition of dilutions of Dex/DMSO or DMSO as described in the results.

RNA Extraction and RT-PCR

RNA was isolated using magnetic Oligo dT Dynabeads (Dynal, Bromborough, UK). Approximately 50 mg of cell pellet frozen in liquid nitrogen were ground in 100 μL of Lysis/Binding buffer (Dynal), centrifuged 1 min at 13,000 rpm in a benchtop centrifuge, then 50 μL of supernatant was added to 20 μL of Dynabeads prepared accordingly to manufacturer's instructions, incubated with gentle agitation for 5 min at room temperature to allow annealing of mRNA to the Oligo dT on the Dynabeads. Dynabeads were separated and washed accordingly to manufacturer's instructions, then resuspended in 20 μL of reaction mixture for RT (Omniscript, Qiagen USA, Valencia, CA) and incubated for 1 h at 37°C. PCR reactions were performed in a reaction volume of 25 μL (Taq Master Mix, Qiagen) with 4 μL of the cDNA reaction mixture containing suspended magnetic beads.

GAPDH amplification was used as loading control. The oligonucleotide primer pairs had the following sequences: *alcR*-forward CTCTAAATCC-TTCGCAACCAGC and *alcR*-reverse GGACGTTTTGGAGAGCATCG for amplification of fragment 400 bp; and *GAPDH* -forward GGTTTGGCA-TTGTGGAGGGTC and *GAPDH*-reverse CCCTCCGATTCCTCCTTGATTGC for amplification of fragment 304 bp. After 15 to 20 rounds of amplification, with primer annealing temperature of 55°C, 10-μL samples of the PCR reaction mixture were separated on a 1% (w/v) agarose gel.

Protein Extraction and Western-Blotting Analysis

Liquid nitrogen-frozen BY-2 cell pellets were homogenized in microfuge tubes in extraction buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM NaF, 0.1% [v/v] Triton X-100, pH 7.5), at a volume of 1 μL/mg of cells; before quantification (Bradford, 1976), 20 μg of soluble extract was loaded per lane. CDKA and GFP proteins were detected on western blots using a mouse anti-PSTAIR monoclonal antibody (Sigma-Aldrich).

GUS Staining

BY-2 cells were suspended in GUS-staining buffer (Jefferson et al., 1987) and incubated overnight at 37°C. GUS-stained cells were washed and suspended in 70% (v/v) ethanol prior to photography. Cells were visualized by microscopy using a Nikon E600 microscope (Tokyo).

GUS and ADH Quantification

For GUS, protein was extracted from liquid nitrogen frozen plant cells/tissue in microfuge tubes by homogenization in GUS extraction buffer (50 mM NaH_2PO_4, 10 mM EDTA, 0.1% [v/v] Triton X-100, 1.0 g L^{-1} Sarcosyl). The GUS activity was determined with a fluorometric assay using 4-methylumbelliferyl β-D-glucuronidase (4MU; Sigma-Aldrich) as a substrate (Jefferson et al., 1987). GUS activity was quantified using either a Perkin-Elmer LS30 (tomato; Perkin-Elmer Applied Biosystems, Foster City, CA) or a Tecan Safire (BY-2) fluorimeter (Zurich) with 365 nm excitation and 455 nm emission wavelengths. ADH activity was determined as described by Rumpho and Kennedy (1981). Total soluble protein was determined as described by Bradford (1976).

Fluorescence Microscopy

The GFP fluorescence of Arath;CDKA;1-GFP and erGFP-expressing BY-2 cells was visualized using a Nikon E600 microscope with excitation with 465 to 495 nm and emission filter 515 to 555 nm.

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