Increased leakiness of the tetracycline-inducible

**Triple-Op** promoter in dividing cells renders it
unsuitable for high inducible levels of a dominant
negative **CDC2aAt** gene

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**Abstract**

A tetracycline-inducible promoter system was used to generate transgenic tobacco plants that confer inducible expression of the wild type or a dominant negative allele of the gene coding for the cyclin-dependent kinase (**CDK**) of *Arabidopsis thaliana** CDC2aAt. Although the total extractable CDK activity was doubled, the induced expression of the wild-type **CDC2aAt** did not correlate with any change of the cell cycle kinetics. An increase of CDK activity upon **CDC2aAt** expression was only seen in dividing cell populations, demonstrating that **CDC2aAt** expression itself is not sufficient to induce CDK activation. Induced expression of the dominant negative **CDC2aAt**.

**N146** correlated with a reduction of CDK activity to 66% of the level found in non-induced cells. This decrease was not sufficient to block cell division. The isolation of plants showing only low inducible levels of **CDC2aAt**.N146 suggests that a counter-selection against strong inducible lines had occurred. Accordingly, **Triple-Op** promoter activity was found in dividing cells in the absence of tetracycline.

Key words: *Arabidopsis thaliana*, cell cycle, chemical-induced expression, cyclin-dependent kinase.

**Introduction**

The use of chemical-inducible promoters allows the study of the effects of lethal genes of which the expression into transgenic lines might otherwise be difficult. For good inducible systems, expression of the transgene should be linked only to the presence of the inducing compound. Furthermore, the inducing compound should not interfere with normal plant development, nor be toxic to the organism. Rapid induction kinetics and an easy application of the inducer are also favourable.

In plants, two major classes of chemical-inducible systems are available (for a review, see Gatz and Lenk, 1998). The first group utilizes sequences of plant origin, such as the promoter of an elicitor, safener, heat shock or wound-inducible gene. The second group of chemical-inducible systems is based on the transfer of well-characterized transcriptional mechanisms from heterologous organisms into plants. This group includes systems induced by copper (Mett *et al.*, 1993), isopropyl-β-D-thiogalactopyranoside (Wilde *et al.*, 1992), ethanol (Salter *et al.*, 1998), ecdysone (Martinez *et al.*, 1999), and glucocorticoids (Aoyama and Chua, 1997). The most successful system belonging to this group is the tetracycline (**Tc**)-inducible promoter (Gatz *et al.*, 1991, 1992). Plants that constitutively overexpress the *Escherichia coli** TetR** repressor gene are transformed with a second construct containing the gene of interest under the control of a **CaMV** 35S promoter with three integrated tet operator sequences (the **Triple-Op** promoter). In the absence of **Tc**, the TetR repressor molecules bind to the tet sequences, which are located around the TATA box, thereby repressing transcription. Upon the application of **Tc**, the TetR repressor molecules are released from the tet operators and transcription is activated. This system has proven to work for the induced expression of the oat arginine decarboxylase (Masgrau *et al.*, 1997), *Agrobacterium rhizogenes** rolB** and rolC (Röder *et al.*, 1997),

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1994; Faiss et al., 1996), and the potato trans-acting PG13 (Rieping et al., 1994) genes. Complementary to the Tc-inducible system, a Tc-repressible system has been developed, using a fusion protein between the TetR repressor and the VP16 activator (Weinmann et al., 1994).

The reported successes of the Tc-inducible promoter prompted the use of this system to study cyclin-dependent kinases (CDKs). CDKs are Ser/Thr kinases that regulate the progression through the cell cycle. Their activity is regulated on the post-transcriptional level by phosphorylation and the association with regulatory proteins such as cyclins, docking factors and CDK inhibitors. In Arabidopsis thaliana, two different CDK genes have been characterized (CDC2aAt and CDC2bAt) (Ferreira et al., 1991; Hirayama et al., 1991). The overexpression of dominant mutant CDC2aAt and CDC2bAt genes has been previously demonstrated to interfere with the cell cycle in yeast (Porceddu et al., 1999). Expression of these mutant alleles in planta would be interesting to study the effects of an aberrant cell cycle on plant development.

This approach was previously used successfully for the expression of the CDC2aAt.N146 (DN) allele. This mutant allele encodes a protein kinase in which the Asp146 residue is mutated into Asn146. The Asp146 residue is conserved over all protein kinases. Three-dimensional structure analysis revealed that this residue is essential for the correct binding of the cofactor ATP (De Bondt et al., 1993), therefore mutating Asp146 generates an inactive kinase. Transgenic tobacco plants that expressed the CDC2aAt.N146 gene displayed a reduced amount of extractable CDK activity, correlated with a decrease in the number of cell divisions, albeit without effect on plant morphogenesis or histogenesis (Hemerly et al., 1995). The dominant effect of the protein is explained by assuming that CDC2aAt.N146 retains its ability to bind to regulatory proteins that are necessary for CDK activity, resulting in a competition between CDC2aAt.N146 and the endogenous CDKs for the same proteins. Studies with an analogous mutation in human CDKs showed that cyclin titration is at least part of the mechanism of kinase inhibition (van den Heuvel and Harlow, 1993).

The regeneration of plants that overexpress CDC2aAt.N146 under the control of the CaMV 35S promoter was found earlier to be problematic (Hemerly et al., 1995). Of many tobacco transformants, only three plants were obtained that expressed the transgene. For A. thaliana no transformants were retrieved at all. This is most probably due to the constitutive expression of the CaMV 35S promoter during the transformation process. Because transgenic plants are regenerated from calli, constitutive expression of a gene blocking cell division will interfere with the regeneration of transformed plants. To circumvent this problem, the mutant CDC2aAt.N146 gene was cloned under the control of the Tc-inducible promoter. This promoter is supposed not to be expressed during the transformation procedure in the absence of Tc. In this way, strong CDC2aAt.N146-inducible lines were expected, which then could be useful for studying the effects of a reduced CDK activity on specific developmental processes, such as lateral root initiation or leaf formation.

Materials and methods

Plasmid constructions and plant transformation

Sequences coding for CDC2aAt and CDC2aAt.N146 were fused to 3’ NOS, cut out of the PH35S vectors (Hemerly et al., 1995) with NeoI and BglII, and cloned blunt-ended into the HpaI and BamHI sites of pUCAT-TX, resulting in the P35STXDC2WT and P35STXDC2D2N vectors. The pUCAT-TX vector contained an expression cassette allowing genes to be placed under the control of the Tc-inducible CaMV 35S (Trip-Op) promoter (Gatz et al., 1992). In parallel, the TXGUS1 construct was obtained by cloning the β-glucuronidase (GUS) reporter gene under the control of the Trip-Op promoter (cut out of pUCAT-TX with EcoRI and Smal) cloned blunt-ended into the NeoI site of pGUS1 (Peleman et al., 1992). The chimeric Trip-Op/CDC2aAt/NOS and Trip-Op/CDC2aAt.N146/NOS genes were cut with EcoRI and XbaI from P35STXDC2WT and P35STXDC2D2N, respectively, and cloned blunt-ended into the Smal site of TXGUS1, resulting in the TXGUS1CDC2WT and TXGUS1CDC2D2N vectors. Subsequently, the whole tandem construct was cut with EcoRI and cloned into the SnaBI site of the PGSC1704 binary vector (a gift from Plant Genetic Systems, Gent, Belgium) to obtain the PGSC-WT and PGSC-DN vectors. The binary constructs were used to transform a Nicotiana tabacum L. plant (var. Wisconsin 38) (a kind gift from Dr C Gatz, Albrecht von Haller Institute of Plant Sciences, University of Göttingen, Germany) that constitutively expressed the TetR repressor (TETREP2) by using the leaf disc transformation protocol (Horsch et al., 1985).

Protoplast preparations

Protoplasts were prepared from leaves of in vitro-grown tobacco plants. Leaves were enzymatically digested overnight with 0.5% cellulase and 0.2% macerozyme in K3 basal medium (Nagy and Maliga, 1976), containing 0.4 M glucose. Protoplasts were cultivated at a density of 10⁵ ml⁻¹ in K3 medium containing 20 g l⁻¹ glucose, 1 ml l⁻¹ α-napthaleneacetic acid, 0.5 mg l⁻¹ 6-benzylaminopurine, and 0.5 mg l⁻¹ zeatin. Cells were cultivated in the dark in the presence or absence of 1.0 mg l⁻¹ Tc (Sigma, St Louis, MO, USA). Cells were harvested by centrifugation at 600 g at the time points indicated in the text, frozen in liquid nitrogen and stored for later analysis.

Protein extraction, CDK kinase assay and protein gel blotting

Protein extracts were prepared by grinding plant material in homogenization buffer (HB) (De Veylder et al., 1997). Kinase assays were performed as described previously (Hemerly et al., 1995), using 30 µl 50% (v/v) SUCI-Sepharose beads and 150 µg protein extract in a total volume of 300 µl HB. For protein gel blots, 20 µg of proteins were separated on a 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electroblotted on nitrocellulose membranes (Hybond-C⁺; Amersham, Aylesbury, UK). Filters were blocked overnight
with 2% milk in phosphate buffered saline (PBS), washed three times with PBS, probed for 2 h with a CDC2aAt-specific (1:5000 dilution) antibody in PBS containing 0.5% Tween-20 and 1% albumin, washed for 1 h with PBS containing 0.5% Tween-20 (PBST), incubated 2 h with peroxidase-conjugated secondary antibody (Amersham), and washed for 1 h with PBST. Protein detection was done by the chemoluminescent procedure (Pierce, Rockford, IL, USA).

Vacuum-infiltration induction experiments

Single leaves from in vitro-grown plants were submersed in a Petri dish containing 10 mg l⁻¹ Tc in 50 mM sodium citrate (pH 5.5) and placed for 5 min in a desiccator. Subsequently, the leaves were incubated in the dark for 2 d in liquid Murashige and Skoog medium containing 10 mg l⁻¹ Tc. Control leaves were treated in the same way, but without adding Tc.

Histochemical GUS staining

Histochemical assays for GUS activity were performed as described previously (Jefferson et al., 1987) with some modifications. Plant material was prefixed with cold 90% acetone for 1 h, washed twice with 100 mM sodium phosphate buffer (pH 7.4), and immersed in the enzymatic reaction mixture containing 0.5 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc; Biosynth, Staad, Switzerland), 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide in 100 mM sodium phosphate buffer (pH 7.4). The reaction was incubated overnight at 37 °C. Visualization of the expression pattern was enhanced by clearing the tissues with lactophenol (Beeckman and Engler, 1994).

Results

Regeneration of transgenic plants

The wild-type CDC2aAt and the mutant CDC2aAt.N146 allele under the control of the Triple-Op promoter were cloned into the PGSC1704 binary vector (see Materials and methods). Because no A. thaliana plants were available that synthesized the TetR repressor, the constructs were used to transform tobacco plants, which constitutively express TetR at levels sufficient to repress the transcription of the Triple-Op promoter in the absence of Tc. After the regeneration of transgenic plants, the strongest inducible lines were selected by protein gel blot analysis using a CDC2aAt-specific antibody (see Materials and methods). During this assay, the number of transgenic plants showing inducible CDC2aAt expression (seven out of 12) was much higher than the number of lines showing inducible expression of CDC2aAt.N146 (four out of 28).

The best inducible CDC2aAt/CDC2aAt.N146 lines were selected for protoplast preparation. Subsequently, the protoplasts were cultivated for 2 d in the absence or presence of 10 mg l⁻¹ Tc. Induction of the CDC2aAt or CDC2aAt.N146 genes was tested by protein gel blotting (Fig. 1). The CDC2aAt/CDC2aAt.N146 proteins could only be detected in the Tc-treated protoplasts, demonstrating the inducibility of the Triple-Op promoter. However, from this experiment, it became clear that the CDC2aAt protein levels exceeded those of CDC2aAt.N146. RNA gel blot analysis presented a similar picture (data not shown), demonstrating that the difference in protein level was not due to the instability of the CDC2aAt.N146 protein.

Optimal induction conditions in mesophyll protoplast cultures

The effects of induced overexpression of CDC2aAt and CDC2aAt.N146 on cell division were studied in protoplast cultures. First, the optimal induction conditions for the Triple-Op promoter in mesophyll protoplasts were determined. The minimal concentration of Tc necessary for induction was measured by the addition of different concentrations of Tc to mesophyll protoplasts prepared from the strongest CDC2aAt-inducible line. Because cell divisions in the experimental system used here started only 3–4 d after preparation of the protoplasts, samples were incubated with Tc for 3 d. The level of CDC2aAt accumulation was investigated by protein gel blot analysis.

No CDC2aAt protein was detected in the absence of Tc or at a concentration of 0.1 mg l⁻¹, but a clear signal was observed at a concentration of 0.5 mg l⁻¹ Tc or higher (Fig. 2). In the following experiments, concentrations of 1.0 mg l⁻¹ Tc were used. Mitotic index determination and [3H]-thymidine incorporation showed that this concentration of Tc did not interfere with protoplast division (data not shown).

![Fig. 1. Comparative levels of protein accumulation in CDC2aAt- and CDC2aAt.N146-inducible lines. Protoplasts of the strongest CDC2aAt- and CDC2aAt.N146-inducible lines were cultivated in the absence (−) or presence (+) of 10 mg l⁻¹ Tc. After 2 d, protein extracts were prepared, 20 µg of protein was separated on a 12% polyacrylamide gel and immunoblotted. Immunodetection was performed with a CDC2aAt-specific antibody. Because the antibody does not cross-react with the endogenous CKDs of tobacco, only the CDC2aAt/CDC2aAt.N146 proteins are detected. The nature of the constitutively present cross-reacting band of 70 kDa is unknown.](image-url)
The kinetics of induction were studied by performing a time course experiment. Mesophyll protoplasts were divided into two populations. One population was incubated with 1.0 mg l⁻¹ Tc. From day 2 onwards a sample of the Tc-treated and non-treated protoplasts was harvested and frozen. After collecting the last samples (at day 5), protein extracts were prepared and protein gel blot was performed. Already after a 2 d incubation, a clear CDC2aAt signal was observed (Fig. 3). A longer incubation resulted in an increase of the amount of CDC2aAt. Remarkably, a significant amount of CDC2aAt protein was also noticed in the protoplast cultures without Tc from day 4 onwards. The level of leakage increased with time, suggesting that the leaky expression of the Triple-Op promoter correlated with the resumption of cell division activity in the protoplast culture.

**Induced expression of CDC2aAt and CDC2aAt.N146 in mesophyll protoplasts**

Mesophyll protoplasts were prepared from the most strongly inducible CDC2aAt and CDC2aAt.N146 plants. As control, protoplasts were prepared from a TetR-synthesizing transgenic plant containing the GUS reporter gene under the control of the Triple-Op promoter. Cultures were divided into two populations, of which one population was incubated with 1.0 mg l⁻¹ Tc. The other was used as control. After 4 d of incubation, cells were harvested and protein extracts were prepared. Using these extracts, the CDK activity was assayed using histone H1 as a substrate (see Materials and methods).

For protoplasts prepared from the control plants, no significant difference in CDK activity was observed between the Tc-treated and control cells, demonstrating that Tc application on its own did not cause any changes in the CDK activity (Fig. 4A). In protoplasts prepared from CDC2aAt-inducible plants, an average increase of kinase activity up to 201% was observed in the Tc-treated cells, compared to that of the non-induced cells. A time-course-dependent induction experiment showed that this increase was observed only after 3 d of cultivation of the mesophyll protoplasts (Fig. 4B), although CDC2aAt expression was already activated from at least day 2 onwards (Fig. 3). The increase of CDK activity at day 3 correlated with the onset of cell division in the protoplast cultures. The observed rise in kinase activity did not result in an increase of the number of dividing cells, in the rate
of onset of cell division or in a change of the G1/G2 ratio, as demonstrated by mitotic index determination and flow-cytometric analysis (data not shown).

In Tc-treated protoplast cultures of CDC2aAt.N146 transgenic plants an average reduction down to 66% of CDK activity was seen, in comparison with non-treated cells (Fig. 4A). However, this decrease was not sufficient to arrest or slow down cell division.

Leakage of the Tc-inducible Triple-Op promoter in dividing cells

The isolation of only a low number of transgenic CDC2aAt.N146-inducible lines suggested that during the transformation procedure a selection against strongly expressing lines had occurred. The leaky expression in dividing protoplasts indicated that this might have happened due to expression of the Triple-Op promoter in dividing cells in the absence of Tc. A significant level of background expression was observed by GUS staining of a tobacco plant transformed with the GUS reporter gene under the control of the Triple-Op promoter. As shown in Fig. 5 (right), seedlings grown at the concentration of 1 mg l⁻¹ Tc displayed a strong induction of GUS activity in the cotyledons and leaves. Expression was also seen in the root apical meristem and the vascular stele (data not shown). GUS staining of a non-Tc-treated seedling revealed a pink colouring in the shoot apical meristem and the flanks of a young leaf (Fig. 5, left). This staining reflects expression of the Triple-Op promoter. A similar background expression was noticed in the root meristem and in calli (data not shown).

Discussion

The Tc-inducible system was used successfully for the overexpression of the wild-type CDC2aAt gene. Induced CDC2aAt expression resulted in a doubling of extractable CDK activity in mesophyll protoplasts. During a time-course experiment, a clear increase of kinase activity was observed only after 3 d of incubation of the protoplasts with hormones, although induced CDC2aAt expression was visible earlier. The doubling of the kinase activity correlated with the onset of cell division in the protoplasts. The observation that CDC2aAt overexpression does not boost the kinase activity in non-dividing cells demonstrates that, besides CDC2aAt, other components are necessary for CDK activation. However, the observed increase of CDK activity from day 3 onwards suggests that once cells are dividing the level of CDK activity is, at least in part, determined by the amount of available CDK protein, meaning that CDK has become a limiting factor.

The observed increase in CDK activity did not result in any change of cell cycle kinetics. Also in HeLa cells and Drosophila no effects on cell cycle progression could be noticed upon CDK overproduction (Krek and Nigg, 1991; Stern et al., 1993). Higher eukaryotes have evolved to use multiple CDKs to regulate progression through the cell cycle (Pines, 1994). Recently, it has been shown that the

Fig. 5. Leakage of the Triple-Op promoter in meristems. The shoot apex of a plant containing the GUS gene under the control of the Triple-Op promoter were grown in the absence (left) or presence (right) of 1 mg l⁻¹ Tc. Expression of the Triple-Op promoter was visualized by histochemical GUS staining using dark-field microscopy.
retinoblastoma protein, an important protein for checkpoint control, needs to be phosphorylated by two distinct CDKs to become activated (Connell-Crowley et al., 1997). Similarly, other substrates may need to be phosphorylated by several CDKs. Therefore, an accelerated cell cycle may require the simultaneous overproduction of different CDKs.

In contrast to CDC2aAt, no plant lines with high inducible CDC2aAt.N146 expression levels could be obtained. In mesophyll protoplast cultures, induced CDC2aAt.N146 expression correlated with a decrease of extractable CDK activity. However, this decrease was not sufficient to inhibit cell division. The plant lines that were obtained might possess the highest level of CDC2aAt.N146 expression attainable without interfering with cell division. The lack of plants with higher expression levels is probably due to the leakage of the Triple-Op promoter in dividing cells. In leaves, no background expression was seen, but as soon as mesophyll protoplasts were stimulated to divide a significant level of leaky expression was noticed. Because transgenic plants were obtained from calli, the leaky expression during the regeneration process might have caused a counterselection against strongly inducible CDC2aAt.N146 lines.

Previously, the construction of a few transgenic plants has been reported that overexpressed a CDC2aAt.N146 construct under the control of the CaMV 35S promoter (Hermerly et al., 1995). The very low frequency by which such transgenic plants were obtained and the low expression levels of the transgenes pointed to a strong counterselection in which only specific construct/genomic context configurations were selected that were compatible with cell division. Because vector and promoter used in the experiments described here are different from those published previously, no direct comparison of the results is possible.

The Tc-inducible system is based upon the competition of the TetR repressor molecules with the proteins that assemble around the TATA box to form a competent transcription complex. Cell division requires the transcription of a lot of different genes. Therefore, the level of bacterial TetR repressor made by the transgenic tobacco lines may not be sufficient to compete with the TATA box-binding proteins in meristematic cells. One way to circumvent this problem might be the regeneration of plant lines that produce more of the TetR repressor. However, this objective could also be problematic as illustrated by the inability to obtain A. thaliana plants with high levels of TetR (Gatz, 1996). Moreover, in tomato, high levels of TetR caused reduced shoot dry weight and chlorophyll content, reduced leaf size, and an altered photosynthetic physiology (Corlett et al., 1996). In conclusion, the Tc-inducible system is not suitable for the overexpression of cell cycle genes that interfere drastically with the process of cell division as long as the problem of leakage of the Triple-Op promoter in meristematic cells is not resolved.

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