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Effect of cadmium treatment on the expression of chimeric genes in transgenic tobacco seedlings and calli

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Abstract. Transgenic tobacco plants and calli bearing the bacterial *uid A* gene under the transcriptional control of *rbcS*, *mas* and CaMV35S promoter(s) were exposed to different concentrations of cadmium. The transcriptional activity of the promoters was monitored using p-nitrophenyl β-D-glucuronide as a substrate for the β-glucuronidase (*uidA*) reporter enzyme. The *rbc S* promoter was repressed by high concentrations of cadmium. An induction of the *mas* promoter was seen after cadmium treatment of seedlings but not calli. The activity of the CaMV35S promoter was unaffected by cadmium in both seedlings and calli.

Key words: Cadmium - Chimeric genes - Transgenic

Introduction

Cadmium is a toxic heavy metal occurring in nature mainly as a trace impurity in copper, lead and zinc ores. During the last century it has been used in many industrial and household applications, thus elevated concentrations are currently found in the environment (Holmgren et al. 1993). Sources of heavy metals in the agricultural soils include such common practices as application of sewage sludge and use of phosphatic fertilizer (Verma and Katz 1978). Phosphatic fertilizers contain 0.1 to 80 mg Cd per kg. If this fertilizer contains 8.0 mg Cd per kg then the top soil is usually contaminated. Twenty mg Cd per kg of soil can occur naturally in mining areas. The problem is enhanced in acid soils as low pH mobilizes cadmium as well as other toxic metals from the soil thereby increasing their availability. Cadmium is readily taken up by plant roots and can have a toxic effect on growth, metabolism and gene expression (Huang et al. 1974; Edelman et al. 1988). Metal toxicity generally causes stunted growth. Tobacco seedlings grown on media containing 50-100 μM $CdCl_2$ show severe root deterioration within two weeks (Misra and Gedamu 1989). Genes involved in the metabolism of phytochelatins and heat shock genes were found to be induced by cadmium (Edelman et al. 1988; Gyorgyely et al. 1991; Ruegsegger and Brunold 1992). The aim of this study was to detect cadmium induced changes on the activity of three promoters, i.e. ribulose bisphosphate carboxylase (*rbc S*), mannopine synthase (*mas* or TR1' 2'), and CaMV35S widely used to direct transcription of alien chimeric genes in transgenic plants, and assess suitability of these promoters to genetically engineer plants for heavy metal tolerance and sequesteration.

The light regulated promoter of the nuclear gene encoding for the small subunit of tobacco ribulose bisphosphate carboxylase (Mazur and Chui 1985) is very active in the photosynthetic tissues of plants. High temperature stress (heat shock) inhibits its activity (Vierling and Key 1985). The dual bi-directional *mas* promoter controlling the production of mannopine synthase (Velten et al. 1984) is widely used in various experiments with transgenic plants. *Mas* is a prokaryotic promoter that contains cis elements for expression and regulation in plants. The promoter is active in many plant tissues and organs, and its expression is enhanced by stress factors, including wounding (Saito et al. 1991) and auxins (Langridge et al. 1989; Leung et al. 1991). Abscisic acid and giberellic acid inhibit the activity of this promoter (Langridge et al. 1989). Very high expression has been recorded in protoplasts and calli (Langridge et al. 1989). The promoter of the Cauliflower Mosaic Virus gene IV (CaMV35S) encoding for the 35S RNA transcript (Odell et al. 1985) is one of the strongest positively-regulated 5' sequences in plants, and has been used to drive expression of alien genes in a broad variety of mono- and di-cotyledonous plant species (Horsch et al. 1984). It is active in most plant organs, although some tissue specificity has been reported. No consistent pattern of induction or repression by growth regulators has been established.

Our current research interest is to genetically engineer plants for heavy metal sequestration and partitioning. In the present work, we optimized and used p-nitrophenyl β-D-glucuronic acid as a chromogenic substrate to analyze the activity of the *rbc S*, *mas* and CaMV35S promoters in transgenic plants and calli in response to cadmium treatment. Our results show that the promoters tested here had different patterns of induction and/or repression in transgenic seedlings and calli treated with cadmium. The significance of these results to the use of these promoters in developing metal tolerant/sequestering transgenic plants is discussed.

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Material and methods

Plasmids and bacterial strains. The plasmids pIS212, pIS312 and pIS412 were based on the binary plant vector pGA482 (An 1986) and were described elsewhere (Stefanov et al. 1994). Fig. 1 shows the genetic maps of the constructs employed in this study. The plasmids were introduced into the disarmed *Agrobacterium tumefaciens* strain LBA 4404 (Ooms et al. 1982) by triparental mating. Confirmed transconjugants were kept as frozen glycerol stocks at -80°C. Before plant transformation the integrity of the vectors was verified by plasmid minipreps and DNA-DNA hybridization with a Hind III- Eco RI fragment containing the *uid* A coding sequence. All molecular biology work was done essentially according to Maniatis et al. (1982) or to the specific manufacturer's instructions.

Plant material, transformation and culture conditions. Axenic Petit Havana SR 1 (tobacco, *Nicotiana tabacum*, Maliga et al. 1973) plants were transformed using a simplified leaf-disc method, as described earlier (Horsch et al. 1984). The putative transformants were selected in Petri dishes with MS (Murashige and Skoog 1962) medium supplemented with the appropriate growth regulators [1.0 mg.L^{-1} benzyladenine (BAP); 0.1 mg.L^{-1} napthaleneacetic acid (NAA)]; 30g.L^{-1} sucrose; 0.8% plant tissue culture grade agar (Sigma); 100 mg.L^{-1} kanamycin and 300 mg.L^{-1} carbenicillin. In order to keep high selective pressure, explants were transferred to fresh medium every two weeks. Kanamycin-resistant shoots derived from discrete transformation events were planted in Magenta jars with hormone-free MS medium supplemented with the same antibiotics and propagated. Plantlets with strong root systems were potted in soil and transferred to a greenhouse. The seeds of self-pollinated plants were collected. In order to ensure a large supply of genetically stable and homogenous material, kanamycin-resistant F2 progeny of self-pollinated representative individual transformants were used for the experiments. Seedlings were grown on hormone-free MS medium. Calli derived from transgenic seedlings were cultivated on MS medium supplemented with 0.5 mg.L^{-1} NAA and 0.5 mg.L^{-1} BAP.

Treatment with cadmium. Axenic three-week-old kanamycin-resistant seedlings were carefully removed from the medium and their roots dipped in Eppendorf tubes filled with sterile distilled water containing 0, 20, 50 and 100 μM $CdCl_2$ for 24 h in a sterile moist environment. Actively growing transgenic calli, about four millimeters in diameter, were incubated with 100 μM $CdCl_2$ and with distilled water as the control for the same time period in the wells of a Falcon tissue culture plate (Multiwell 3047). Ten seedlings and two calli from each transformant were used in each of the experimental conditions. All experiments were performed in triplicate.

β-Glucuronidase assays. Samples were prepared essentially as described previously (Stefanov et al. 1994). Twenty microliters of the cell extracts were mixed thoroughly in 1ml assay buffer preheated to 37°C containing 1mM substrate p-nitrophenyl β-D glucuronide (Sigma N-1627). Incubation was performed in spectrophotometric cuvettes (Sarsted No 67.742) in an incubator. Reactions were stopped with the addition of 400 μl stop solution - 2.5 M 2-amino-2-methyl 1-3 propanediol (Sigma A 9754) with thorough mixing. The course of the reactions was monitored by comparing the absorption of reactions stopped at time 0, 60, and 120 min. Short assays were used to minimize the possibility of microbial contamination and/or degradation of the enzyme. Extreme care was taken to ensure uniformity of the treatment for all samples. Measurements were taken with a Beckman DU-65 spectrophotometer calibrated with a mixture of 1ml assay buffer and 0.4 ml stop solution. The resulting absorptions were translated into product concentrations using a standard curve. Specific activity was calculated as units β-glucuronidase activity per milligram total protein. A unit of GUS activity is the amount of enzyme producing 1 nanomole of p-nitrophenol (pNP) per minute at 37°C. All experiments were performed in triplicate.

Results and discussion

Production of transgenic plants

Thirty two GUS-positive tobacco plants bearing the CaMV35S -GUS, 12 plants containing the *rbc* S -GUS and 45 with the *mas*- GUS construct were regenerated and representative plants were selected. Because of the sensitivity of the assay, the rather large variation in activity between transformants, and the need to register small changes, we used individual plants instead of pooling several plants in each assay (An 1986). Representative plants were selected as those having an activity closest to the mean of all GUS-positive plants bearing the same construct.

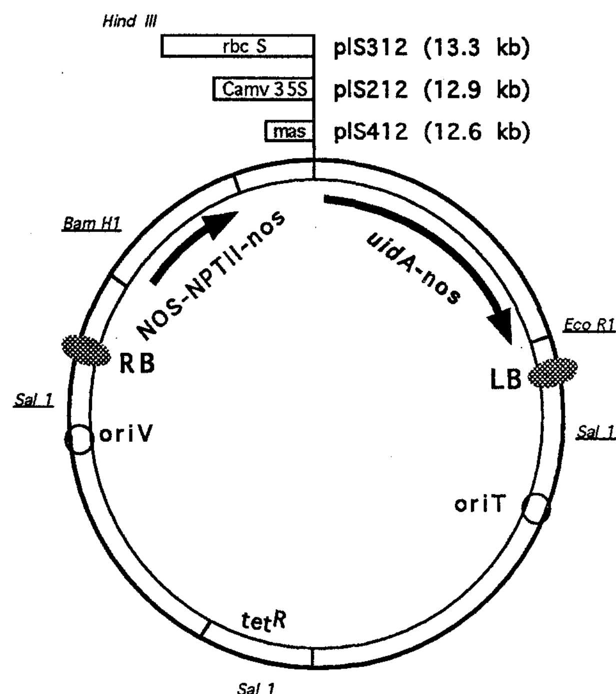

Fig.1. Chimeric gene constructs. LB and RB indicate the left and right T-DNA borders respectively; NOS- nopaline synthase promoter; rbc S-ribulose-1,5-bisphosphate carboxylase promoter; CaMV 35S-cauliflower mosaic virus gene IV promoter; mas- mannopine synthase promoter; nos- nopaline synthase termination signal; NPT II- neomycin phosphotransferase coding sequence; *uid A*- β-glucuronidase coding sequence; tet R- dominant gene conferring bacterial resistance towards tetracyclin; ori T- origin of conjugal transfer; ori V- origin of replication of the broad-host range plasmid pRK2

Optimization of chromogenic uidA assay for promoter activity

The transcriptional activity of the promoters was quantified from the enzymatic activity of the reporter protein using the chromogenic substrate p-nitrophenyl β-D-glucuronide (pNPG). The substrate is cleaved by the enzyme to produce p-nitrophenol, thus shifting the absorption maximum of the assay mixture to 415 nm (Jefferson 1987; Naleway 1992; Wilson et al. 1992). Although the use of pNPG as a substrate for quantitative determination of GUS activity in plants was mentioned in Jefferson (1987), it has scarcely been used (e.g. Wozniak and Owens 1994). We optimized the experimental conditions to test the reliability and suitability of this assay system. Results showed that the concentration-dependent absorption of the pNPG assay mixture was linear up to 60μM p-nitrophenol (Fig. 2A). It was linear to a molarity of about one order of magnitude higher than the linearity of the assay with MUG. Therefore, no dilution was necessary for samples with high activity when using the pNPG assay system. A crucial factor during the analysis of a large quantity of samples was to ensure uniform conditions for all samples. In order to do this and to save time assays were performed directly in the

spectrophotometric cuvettes and the results compared with those obtained with the method suggested by Jefferson (1987). The results demonstrated that if care was taken to preheat all the components of the system, and not to allow the temperature to drop, the two methods gave similar results (data not shown).

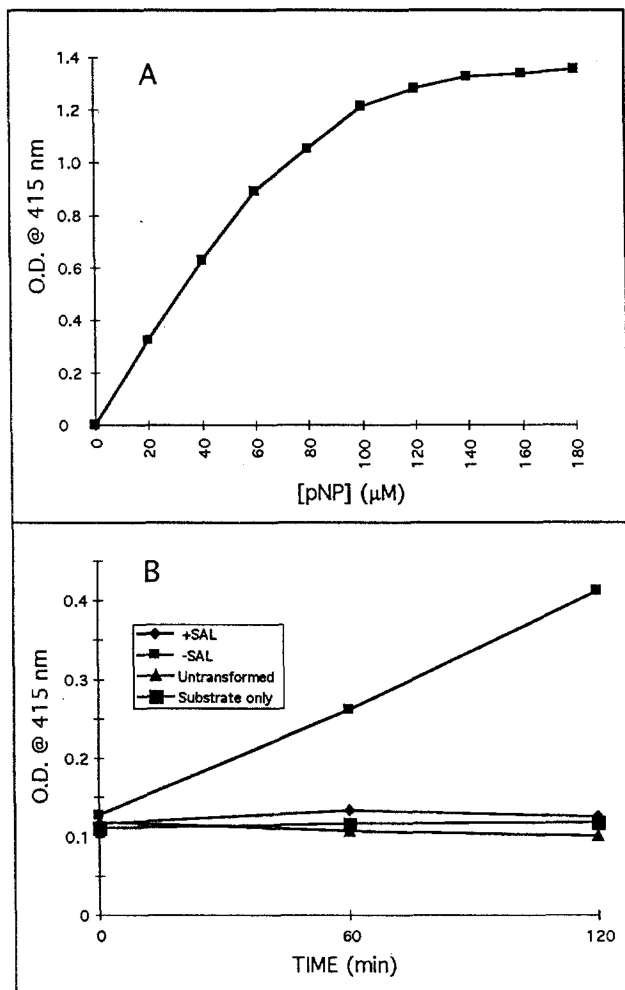

Fig. 2. (A) Linearity of the GUS assay with pNPG as a substrate, and pNP as a product. (B) Inhibition of β-glucuronidase activity in plant extract by 1mM saccharic acid lactone (SAL).

Recently, the presence of GUS-like activities in plant samples was reported by several authors (Hu et al. 1990; Martin et al. 1992; Wozniak and Owens 1994). To detect any contaminating GUS-like activities in tobacco samples, the following two tests were employed: **1. Electophoretic separation of proteins with *in situ* detection of enzymatic activity (zymogram).** Crude protein preparations from selected plants were separated by gel electrophoresis and stained for GUS activity. The limits of detection were established by separating 0.03, 0.15, 0.3, 0.4 and 0.5 units from a crude plant extract with a Mini Protean gel apparatus (Bio Rad Laboratories) and subsequent staining with: a) MUG (Sigma M 9130) according to Martin et al. (1992). b) 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc, Sigma B 3783). c) naphthol AS-BI β-D-glucuronide (Sigma N 1875) and post-coupling with Fast garnet GBC (Sigma F 8761), at pH 7 essentially according to Kyle et al. (1992). In all experiments the procedures revealed a single high molecular weight band with β-glucuronidase enzymatic activity (data not shown). No band was observed in control non-transformed plants. **2. Specific inhibition of GUS activity.** The addition of 1mM D-saccharic acid 1,4-lactone (SAL, Sigma S0375, Jefferson 1987) resulted in complete inhibition of the reaction, indicating a single β-glucuronidase activity, present only in transgenic plants (Fig. 2 B).

The above results ensured that no endogenous or contaminating GUS-like activities were present in the samples and any change in GUS activity following treatment of plantlets or calli was the result of changes in the transcriptional activity of the chimeric promoters.

The GUS activity in control (water-only treated) seedlings and calli was approximately the same as the activity of plants assayed immediately after removal from the original medium (data not shown). This proved that the handling itself was not stressful for the plantlets or the calli and the expression of the chimeric genes was not altered.

Effect of exposure to cadmium on the expression of chimeric genes in seedlings and calli

The three promoters tested here for response to cadmium treatment had different patterns of induction or repression in seedlings and calli (Fig. 3 A-B). The mannopine synthase promoter was induced by cadmium in seedlings in a concentration-dependent manner (Fig. 3A). These results support earlier reports for stress-induced expression of this promoter (Saito et al. 1991), and add cadmium to the list of inducers. In calli, the basal level was very high, so changes in activity induced by cadmium could not be detected (Fig. 3B).

The CaMV35S promoter had a broad range of fluctuation in seedlings and it was not possible to find a statistically-significant concentration-dependent change in GUS activity. Nevertheless, based on experiments-with calli and seedlings, we conclude that 100 μM cadmium had little effect on the activity of CaMV35S promoter.

As seen in Fig. 3A, low, non-phytotoxic concentrations of cadmium (Misra and Gedamu 1989) did not affect the activity of the *rbc S* promoter in seedlings, at least for the short period of treatment. However, higher concentrations (50-100 μM) of $CdCl_2$ had a definite inhibitory effect on the transcriptional activity of the *rbc S* promoter. In control and cadmium treated calli the promoter was inactive (Fig. 3B).

An additional experiment was performed, using the two human metallothionein promoters hMT IIA and hMT IG (Jahroudi et al. 1990) fused to the *uid A* gene. We could not detect any cadmium induced changes of MT promoter activity in transgenic tobacco plants and in calli (data not shown). These results confirm previous observations that mechanisms of MT gene activation by metals in plants is different from that in mammalian cells (Pautot et al. 1989). Recently, it was shown that the yeast MT promoter which is inducible by copper could be activated in plants when the cDNA of the regulatory protein is also introduced in plants (Mett et al. 1993).

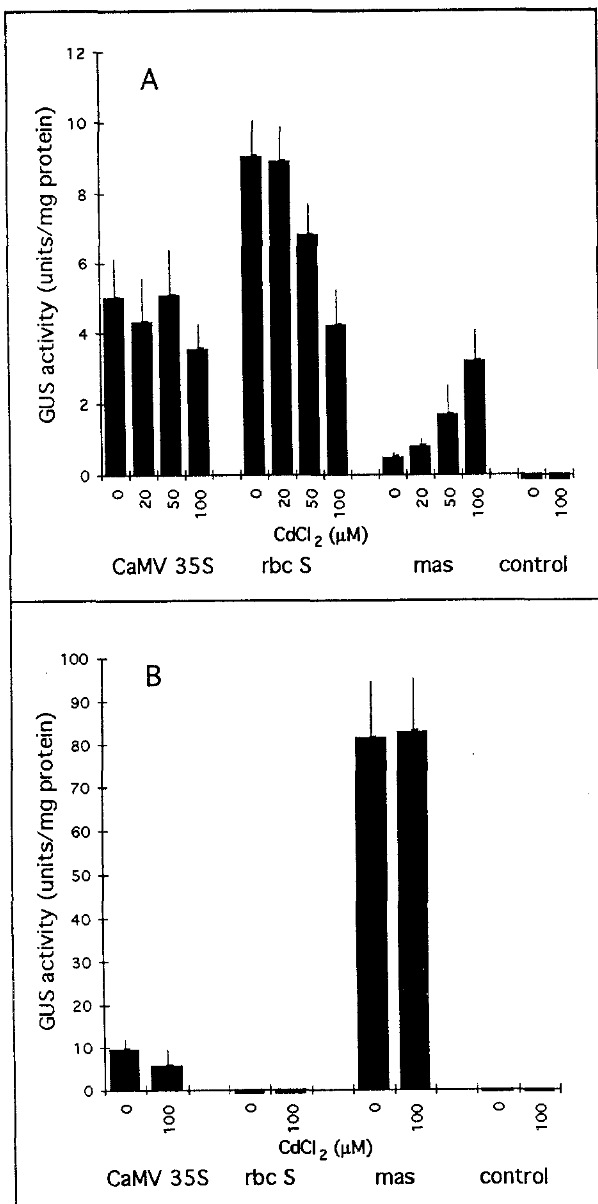

Fig. 3. Effect of cadmium treatment on the expression of chimeric genes in (A) seedlings and (B) calli. Ten seedlings and two calli from individual transformants were used in each experimental condition. All experiments were performed in triplicate.

Transgenic technology has been applied with success in engineering resistance to herbicides (Stalker et al. 1988; Fillati et al. 1987), insects (Vaeck et al.) and viruses in plants (Powell-Abel et al. 1986; Harrison et al. 1987). Manipulating the expression of metal binding proteins/peptides in plants could reduce the dietary intake from crop plants by partitioning toxic metals to inedible plant parts (Misra and Gedamu 1989). Conversely, engineering plants with high metal accumulation in the harvestable aerial parts may increase the effectiveness of phytoremediation of polluted soils. Our results indicate that some restrictions may apply in the use of promoter sequences when plant systems are being designed for sequesteration and partitioning of toxic metals in transgenic plants. For example, the rbcS promoter may be repressed by high concentrations of cadmium, thereby reducing its effectiveness in partitioning of toxic metals in leaf tissues. The *mas* promoter, however, is induced by cadmium, indicating its potential use for the expression of metal-binding proteins and peptides. In summary, based on our data, the CaMV35S promoter presently in use for expressing metal-binding proteins in plants, and the cadmium-inducible *mas* promoter may be suitable for engineering metal tolerant and/or sequestering plants.

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