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SHORT COMMUNICATION

COMPLEX BEHAVIOR OF A COPPER-INDUCIBLE GENE EXPRESSION SYSTEM IN TRANSGENIC POPLAR

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ABSTRACT

We studied a copper-inducible gene expression system first demonstrated in transgenic tobacco for its function in poplar (genus *Populus*). The system consists of a constitutively expressed, copper-activated transcription factor (*ACE1*), together with a hybrid promoter containing an ACE1 binding site (MRE) and the –90 fragment of the cauliflower mosaic virus 35S promoter (CaMV/35S/-90), both driving the *GUS* (β-glucuronidase) reporter gene. A control construct lacked the *ACE1* gene. Surprisingly, *GUS* expression occurred at high levels in the absence of the *ACE1* gene in transgenic poplars. When the *ACE1* gene was present, *GUS* expression occurred even without added copper ion, and showed a parabolic dependence on copper concentration up to 50 μM, above which it inhibited expression. The system does not appear to provide useful copper-inducibility in poplar, possibly due to an endogenous factor that interacts with *ACE1* and high levels of basal transcription from the minimal 35S promoter used.

Keywords: *Populus*, *ACE1*, copper-inducible, MRE, transgenic poplar.

INTRODUCTION

Several chemical-inducible promoters have been identified from plants, lower eukaryotes and prokaryotes (reviewed in GATZ & LENK 1998, JEPSON *et al.* 1998). Copper has been considered to be a useful chemical for inducing gene expression in plants because it is non-toxic at low concentrations and is readily absorbed by plants. However, as an essential element it may also be affected by endogenous copper and changes in physiology that affect copper metabolism (METT *et al.* 1993). To assess the general utility of a copper-based gene induction system in plants it is therefore important to verify its function in diverse species.

METT *et al.* (1993) described a copper-dependent regulatory construct adapted from the yeast copper-metallothionein system for controlling gene expression (Figure 1). Copper binds to *ACE1*, causing a conformational change in the protein and thus enabling copper-dependent transcriptional activation of its target promoter. The system was used to provide copper-inducible cytokinin production in tobacco (MCKENZIE *et al.* 1998), and modified to provide a tissue-specific copper induction in *Lotus* (METT *et al.* 1996).

Previous analyses showed that when tobacco plants were transformed with a control construct, "contruct 3" (CT: pMB 705) which contained a hybrid promoter (MRE::CaMV/35S/–90) directing expression of the *GUS* reporter gene but lacking the *ACE1* gene, *GUS* expression was not detected, even in the presence of copper (METT *et al.* 1993). However, *GUS* expression was induced by copper in transgenic tobacco transformed with the full construct, "construct 61" (FT: pMB711), which contained both the hybrid promoter and the constitutively expressed CaMV/35S::*ACE1* gene.

To evaluate the potential of the copper-controllable gene expression system to direct transgene expression in poplars, we tested the system of METT *et al.* (1993) in hybrid cottonwood. Contrary to expression in other plants, we report that the expression system was activated in absence of both the exogenous copper and the ACE1 activating protein.

MATERIALS AND METHODS

A hybrid cottonwood clone 189-434 (*Populus trichocarpa* × *P. deltoides*) was transformed with either the CT or FT binary plasmids (P. Reynolds, The Horticulture and Food Research Institute of New Zealand). Transformation was carried out using an *Agrobacterium*-based method that has been used in our laboratory for a number of years to produce phenotypically normal trees that stably express transgenes (HAN *et al.* 2000, MOHAMED 1999: http://www.fsl.orst.edu/tgerc/strauss/mohamed-thesis-99.pdf). Transgenic plants were selected based on rooting ability

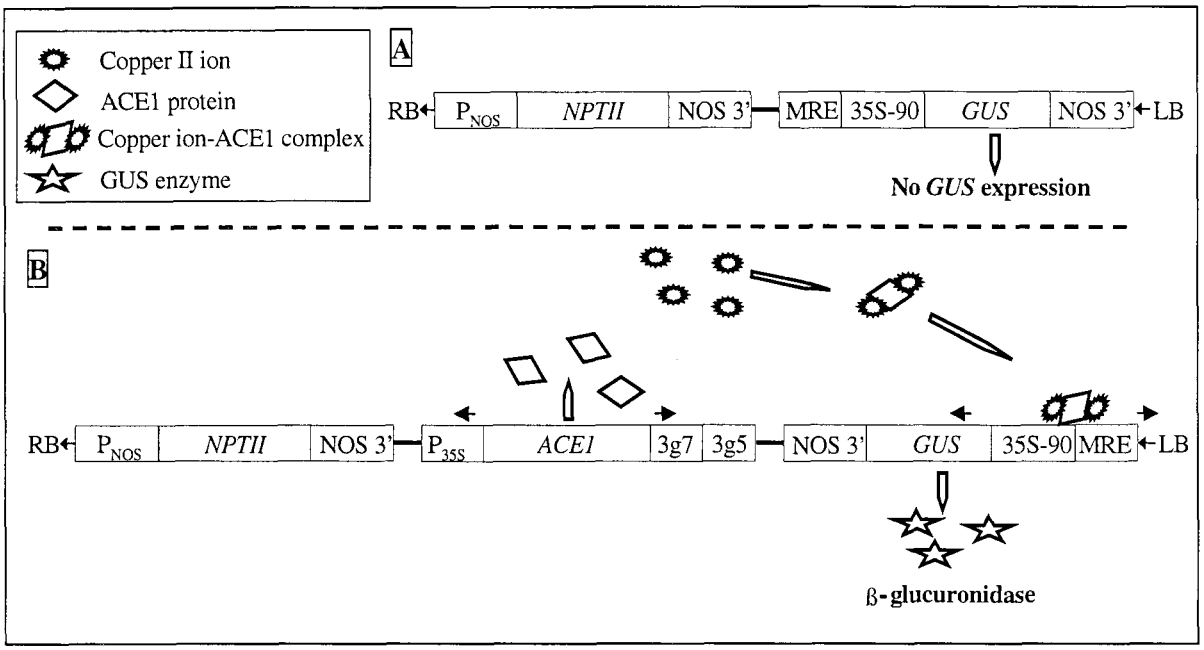

Figure 2: The idealized mechanism of action of a copper-inducible gene expression system (after METT *et al.* 1993). Panel A is a schematic diagram of the control construct (CT, pMB705) and panel B of the full construct (FT, pMB711). The pMB705 and pMB711 are respectively "construct 3" and "construct 61" as referred to by METT *et al.* (1993). In the presence of copper II ion and *ACE1* gene (panel B), the ACE1 protein binds copper and associates with the transcription factor-binding site (MRE), stimulating transcription. In the absence of the transcription factor, *ACE1* gene (panel A), the MRE element could not trigger *GUS* expression. P_{35S} = CaMV 35S promoter; *NPTII* = neomycin phosphotransferase II (kanamycin resistance gene); NOS 3' = polyadenylation terminator signal; 35S-90 = 90-bp domain A of the CaMV 35S; *GUS* = β-glucuronidase; *ACE1* = activating copper-metallothionein expression; P_{NOS} = nopaline synthetase promoter; 3g7 and 3g5 = terminator gene 5 and gene 7 from *Agrobacterium tumefaciens*. RB and LB = right and left borders of the binary vector, respectively. Bulleted arrows (→ ←) indicate relative positions of primer pairs used at amplifying the two critical controlling regions as described in Materials and Methods.

in the presence of 25 mgL^{-1} kanamycin.

For PCR analysis, genomic DNA was isolated from two- to three-month old plants growing in tissue culture, utilizing a modified version of the mini-scale DNA preparation protocol (http://www.fsl.orst.edu/ tgerc/-dnaext.htm). Transformant lines regenerated from different calli were verified through PCR amplification of *NPTII* (kanamycin resistance gene) and *ACE1* genes from plants transformed with constructs CT and FT, respectively. The primer sequences for amplifying *NPTII* were:
forward primer 5' TTCGTCCAGATC ATCCTG 3', and reverse primer 5' TTCTTTTTGTCAAGACCG 3', which amplified a band of 343 bp. The primer sequences for amplifying the *ACE1* were: forward primer 5' CACACTGATGGTCCGCTA 3' and reverse primer 5' CAATATCGTTTAGTGCTGTGTTC 3', which amplified a band of 532 bp.

To verify the integrity of transgenes in our transgenic poplars, we employed the primer pairs
5'CCATCATTGCGATAAAGG 3' and
5' CGTAAATTGATGATATAGCTAGC 3' (966 bp) to amplify across the CaMV/35S::*ACE1* fusion, and
5' GGTACCCGGGGATCCTCTAG 3' and
5' CCAGACTGAATGCCCACAGGCC 3'(272 bp) to amplify across the MRE::CaMV/35S/-90::*GUS* fusion (Figure 1, panel B) on genomic DNA samples of the two intensively studied FT transformants, lines 41 and 47.

GUS activity was assayed using histochemical and fluorometric methods (JEFFERSON 1987). For the histochemical assay, leaf sections (approximately 1.0 × 0.6 cm) were excised from one- to two-month old plants propagated in ½-MS (Murashige and Skoog) medium (Gibco, BRL: 0.032 μM $CuSO_4$). Duplicated leaf samples from separate plants were placed at five different $CuSO_4$ concentrations in distilled water (0, 5, 25, 50 and 100 μM) and allowed to stand overnight. Histochemical GUS expression was scored qualitatively after explants were incubated for 24 hours at 37 °C and cleared in 70% ethanol. For the fluorometric assay, a total of 30-leaf sections were excised from 15 plants, mixed and randomly placed in triplicates in a range of $CuSO_4$ concentrations (0, 5, 25, 40, 50, 60, 70, 80, 90 and 100 μM) followed by protein extractions. Extracts' protein concentrations were determined using a BioRad

Protein Assay Kit, and fluorometric *GUS* activity (JEFFERSON 1987) calculated as 4-methyl-umbelliferone (MU) per mg protein per 30-min incubation at 37 °C. Protein content and fluorometric activity were both measured three times.

The response of transgenic and non-transgenic plants to copper uptake in whole-plant induction assays was investigated by regenerating plants in liquid ½-MS medium, transferred to Cu-depleted medium (distilled water) for three days, then grown for five additional days in ½-MS medium supplemented with 50 μM $CuSO_4$. By comparison, 0.15 μM $CuSO_4$ was present in the copper-depleted medium of METT *et al.* (1993). Leaf sections were then prepared as above for direct histochemical or fluorometric assays.

RESULTS

Seven independent (regenerated from different calli) CT lines, and 10 FT lines, were produced. Transformed lines were verified through analysis of GUS activity, and by PCR amplification of *NPTII* and *ACE1* genes. The integrity of transgenes in plant genome was demonstrated through sequenced junction fragments of amplified sections of the CaMV/35S::*ACE1* and the MRE:: CaMV/35S/-90::*GUS* gene fusions. Sequence comparisons of these critical regions for gene regulation, between two FT transgenic poplar lines (41 and 47) and the binary vector (pMB711), to the reference sequences in GenBank confirmed their integrity.

Based on histochemical GUS assay, the CT lines exhibited high levels of GUS activity regardless of the $CuSO_4$ concentration, including the 0 μM copper control. At any given copper concentration (0, 5, 25, 50, 100 μM), staining intensity was higher in all of the CT lines than was observed in any of the FT lines, based on visual examination. *GUS* activity was not observed in non-transgenic plants. Interestingly however, *GUS* expression varied as a function of copper concentration in the eight FT lines (of a total of 10 produced) that showed detectable levels of *GUS* expression. Addition of 5 or 25 μM $CuSO_4$ decreased GUS staining compared to the zero copper control. The highest levels of *GUS* activity occurred at 0 and 50 μM. Exposure of FT tissue to 100 μM $CuSO_4$ greatly inhibited *GUS* staining. Two of the FT lines (41 and 47) that had strong *GUS* expression were studied over additional copper concentrations (60, 70, 80 and 90 μM $CuSO_4$). In both lines, staining intensity decreased above 50 μM.

Because 50 μM seemed to be the cut-off point above which staining decreased, we induced copper uptake *in vivo* by growing plants in copper-supplemented medium for five days. Histochemical staining again revealed high levels of *GUS* activity in all CT lines, grown with or without added 50 μM copper. No background *GUS* activity was observed in non-transgenic plants. FT lines displayed strong *GUS* staining when induced with 50 μM copper and when no copper was added. There was no evidence that copper induction influenced GUS activity differently between CT and FT groups ($p<0.05$, non-parametric method, SNEDECOR & COCHRAN, 1967) even when copper was given at the optimal inductive conditions of five days, as suggested by METT *et al.* (1993).

The levels of *GUS* expression were quantified in two FT lines, 41 and 47, via fluorometric GUS analysis (Figure 2). In both transgenic lines, copper concentration affected GUS activity in a very similar manner to what had been observed in the histochemical studies. The highest activities were seen at 0 (277±47.9; mean ± one standard error) and 50 μM $CuSO_4$ (351 ±79.1). Low levels of copper (5–40 μM) inhibited GUS activity, as did levels of 60 μM and above. Activity was 12-fold higher at 50 μM than at 100 μM.

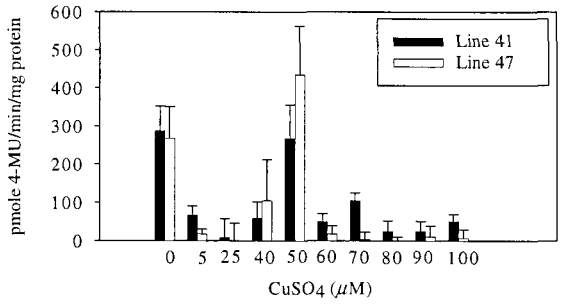

Figure 2: Effect of copper ion concentration on *GUS* gene expression via fluorometric assays. Two independent transgenic poplar lines (*P. trichocarpa* × *P. deltoides*, clone 189-434), that contained the full copper-induction gene construct (FT), are shown. One standard error of the mean from three replicates is shown above each bar. Data are from the difference between expression levels of transgenic and non-transgenic control plants.

DISCUSSION

In tobacco CT lines, GUS activity was not observed under any circumstances (METT *et al.* 1993). By contrast, strong constitutive *GUS* expression was observed in seven independent poplar CT lines in both the presence and absence of copper. The CT construct lacks the *ACE1* gene, which was expected to be necessary for induction of the copper-regulated promoter. Thus, it appears that the *ACE1* protein is not required for transcription from the MRE-minimal 35S promoter in poplar.

Constitutive *GUS* expression of the CT lines could result from an endogenous, copper-independent transcription factor in poplar that binds to and activates the MRE in a manner analogous to that of the activated

ACE1 protein. The hybrid promoter contains an Activating Sequence Factor 1 (ASF 1) binding site in the CaMV 35S -90 promoter fragment. In tobacco, constitutive *GUS* expression was detected in transgenic roots, but not in leaves (METT *et al.* 1996). This may be a result of the CaMV 35S -90 fragment targeting gene expression predominantly in meristematic root tissue of tobacco (BENFEY *et al.* 1989). In poplar, however, this region may have caused more generalized expression. Alternatively, an endogenous poplar factor may have bound to another segment of the hybrid promoter to constitutively activate expression. Plants such as pea (EVANS *et al.* 1990), soybean (KAWASHIMA *et al.* 1991) and maize (DE FRAMOND 1991) contain native metallothionein-like proteins.

In the FT lines, the 60 to 100 μM range may have caused copper toxicity, similar to results with prolonged growth of tobacco at 50 μM $CuSO_4$ (METT *et al.* 1993). The complex pattern of induction seen between 0 and 50 μM may be the result of an endogenous poplar factor that binds at or near the MRE. Binding appeared to be independent of added copper, as evidenced by the lack of dependence of *GUS* expression on copper in the CT transgenic poplars. However, active Cu::ACE1 protein complexes that form in the FT plants at low copper concentrations may compete or interact with the endogenous factor, possibly forming an inactive heteromeric complex. This could cause the reduction in expression observed following addition of 5–40 μM copper. At 50 μM, there appears to be sufficient Cu::ACE1 to outtitrate the endogenous factor and fully stimulate transcription. In *Arabidopsis*, a single transformant containing the FT construct was observed to constitutively activate reporter gene expression (V. METT, personal communication) in a fashion similar to that in poplar, *i.e.* high levels of expression independent of copper. This single transformant may have been the result of the full construct being integrated within a constitutively active segment of the genome, thus exceeding the hybrid promoter-controlling power over the reporter gene.

The system we tested does not appear to be useful as a simple means for control of gene expression in poplar. METT *et al.* (1996), however, later described a version of the copper-regulated promoter in which the CaMV 35S -90 promoter fragment was truncated to -46, eliminating the *ASF-1* binding sites and maintaining just the TATA box-containing fragment of the 35S promoter. Additionally, four repeats of the MRE, instead of one, were fused to the CaMV 35S -46 promoter fragment. With the new construct, constitutive background *GUS* expression in roots was reduced (METT *et al.* 1996). This system should also reduce background expression in the absence of *ACE1* in poplar, but may still give a complex induction pattern if ACE1 indeed interacts with an endogenous factor.

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