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BRIEF COMMUNICATION

# Preparation of vectors with metallothionein gene enriched by additional metal binding domain and their transient expression in *Nicotiana tabacum*

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## Abstract

The issue of finding plants suitable for phytoremediation of inorganic contaminants can be addressed through the preparation of genetically modified plants with an increased metal accumulation potential. A *HisCUP* gene, which encodes for a yeast metallothionein fused with a polyhistidine tail (His), was chosen for preparation of two plant vectors. These two plant vectors were constructed and a *HisCUP* gene expression was subsequently investigated. We firstly prepared a vector pNOV2819/RbcS/*HisCUP* which enabled selection on a mannose medium and contained the *HisCUP* gene under an inducible Rubisco promoter. Secondly, we designed a vector pGreen0029/35S/*HisCUP* which enabled selection of plants on a medium with kanamycin and carried the *HisCUP* gene under a constitutive CaMV 35S promoter. The transient expression of the *HisCUP* gene in tobacco plants was confirmed at RNA and protein levels for both constructs. The relative expression of the *HisCUP* gene was determined by semi-quantitative real-time PCR; a higher expression was detected for the vector pNOV2819/RbcS/*HisCUP*.

*Additional key words*: heavy metals, *HisCUP*, phytoremediation, RT-PCR, tobacco.

Conventional decontamination methods for soils polluted with heavy metals are very expensive and can also negatively affect soil quality (Cherian and Oliveira 2005). Phytoremediation is one biological method which has a high potential for decontamination of soil polluted with heavy metals (Macková *et al.* 2006). Phytoextraction (a type of phytoremediation) involves the uptake and accumulation of pollutants into plant biomass from the contaminated environment. Plant species useful for metal phytoextraction are expected to absorb metals from soil and efficiently translocate and accumulate them in easily harvested shoots (Cunnigham and Ow 1996, Guo *et al.* 2014). Plants known as hyperaccumulators can take up one or more metal/metalloids to concentrations greater than 50 - 100× those of the surrounding vegetation or 100 - 10 000 mg $kg^{-1}$(d.m.), depending on the element (Cappa and Pilon-Smits 2014). Although these plants can hyperaccumulate potentially toxic metals, the majority of these plants have slow growth, low biomass production, ability to accumulate only one specific metal, and habitat specificity. These potential limitations can be mitigated by the preparation of genetically modified (GM) plants with a faster potential growth and an enhanced capability to accumulate heavy metals (Shah and Nongkynrih 2007, Macek *et al.* 2008). The introduction and overexpression of genes for metal transporters (Bhuiyan *et al.* 2011) or metal chelating ligands (Reisinger *et al.* 2008) to plants with fast growth rates has shown to be a promising approach to enhance heavy metal accumulation. Modified plants could have a higher capability of metal accumulation or a higher resistance to a toxic metal. The accumulation of metals can be further increased using plant growth regulators or chelating agents (Bulak *et al.* 2014).

Metallothioneins (MT) are ligands with a high affinity for heavy metals (Kotrba *et al.* 2009). An additional metal binding domain can be added to the MT-molecule to improve metal remediation capabilities. This potential

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*Abbreviation*: CaMV 35S - cauliflower mosaic virus promoter, cDNA - complementary DNA, Ct - cycle threshold, GM - genetically modified, GUS - β-glucuronidase, MT - metallothionein, Rubisco - ribulose-1,5-bisphosphate carboxylase-oxygenase.
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led to the preparation of a genetic construct which allows the expression of *MT CUP1* from yeast *Saccharomyces cerevisiae* associated with a polyhistidine tail (His) (Macek *et al.* 1996), and the positive effect on heavy metal accumulation was proved in GM tobacco (Macek *et al.* 2002). The aim of the present study was to prepare new plant vectors containing a *HisCUP* gene (accession number KJ815139) under different promoters or with different selection systems of GM plants and to confirm their transcription and translation in plants. The combination with the highest remediation potential could, in future, serve for preparation of a transgenic industrial crop, *e.g.*, flax (*Linum usitatissimum*) to allow more economically feasible phytoremediation.

All plasmids in this work were maintained and multiplied in an *Escherichia coli* strain DH5α into which the plasmids were inserted by a heat shock method (Sambrook and Russell 2001). For transient expression, an *Agrobacterium tumefaciens* strain C58-C1 (pCH32) was used (Nováková *et al.* 2009) and the plasmids were introduced into these bacteria by electroporation. *Nicotiana tabacum* L. cv. Wisconsin 38 plants were grown in pots with soil in a cultivation room at a temperature of 24 ± 1 °C, a relative humidity of 40 %, a 16-h photoperiod, and an irradiance of 50 μmol $m^{-2}$ $s^{-1}$ (provided by cool white fluorescent tubes).

For preparation of a pNOV2819/RbcS/*HisCUP* vector, the *HisCUP* gene was amplified by PCR with modified primers HisCUP/F/NcoI (5'-CTC ATC GCC ATG GGG GGT TCT CAT CAT CAT C-3') and HisCUP/R/BglII (5'-CAG CCG AAG ATC TTC TCA TTT CCC AGA GCA GCA TG-3') containing recognition sites for *Nco*I and *Bgl*II restriction enzymes at their 5`-ends. A pTrc*HisCUP* plasmid (Truksa *et al.* 1996) was used as template DNA. The RbcS cassette with the promoter and terminator of Rubisco was obtained from a pIV1.1 plasmid (provided by *Plant Research International*, Wageningen, The Netherlands) by cutting with *Hin*dIII and *Pac*I enzymes and inserting into a pNOV2819 plasmid (provided by *Syngenta*, Basel, Switzerland). The obtained pNOV2819/ RbcS construct was digested by *Nco*I and *Bgl*II enzymes in the RbcS cassette and ligated with a *Nco*I/*Bgl*II digested *HisCUP* amplicon. The presence of the *HisCUP* gene in the construct was confirmed by PCR with specific primers HisCUP/F (5'-CATCATGGTATGGCTAGCATGACT GG-3') and HisCUP/R (5'-TCATTTCCCAGAGCA GCATGAC TTC-3'). The final construct was checked by a restriction cleavage with *Hin*dIII and *Pac*I enzymes.

For preparation of a pGreen0029/35S/*HisCUP* vector, the *HisCUP* gene was amplified using primers HisCUP/F/HindIII (5'-GTA GCC AAG CTT GGA TGG GGG GTT CTC ATC ATC-3') and HisCUP/R/EcoRI (5'-GCT AGG AAT TCC TCA TTT CCC AGA GCA GCA TG-3') containing recognition sites for *Hin*dIII and *Eco*RI restriction enzymes. The construct pNOV2819/ RbcS/*HisCUP* was used as template DNA. The amplicon was digested with *Hin*dIII and *Eco*RI enzymes and ligated with a *Hin*dIII/*Eco*RI digested pSK/35S plasmid (www.pgreen.ac.uk) offering the promoter (CaMV 35S) and terminator regions of cauliflower mosaic virus. The 35S/*HisCUP* cassette was cleaved out from obtained pSK/35S/*HisCUP* by *Eco*RV and ligated with an *Eco*RV digested pGreen0029 plasmid (www. pgreen.ac.uk). The presence of the *HisCUP* gene in the vector pGreen0029/35S/*HisCUP* was confirmed by PCR with primers HisCUP/F and HisCUP/R. The final construct was checked by a restriction cleavage with *Hin*dIII, *Eco*RI, and *Bgl*II enzymes. The sequences of the *HisCUP* gene in both the prepared plasmids were verified by sequencing (*Life Technologies*, Foster City, CA, USA).

The prepared plasmids were introduced into the *A. tumefaciens* strain C58-C1 (pCH32) using a *MicroPulser* device (*Bio-Rad*, Herkules, CA, USA) as per manufacturer's instructions. The pGreen0029/35S/ *HisCUP* construct was co-transformed into bacterial cells together with a plasmid pSoup (www.pgreen.ac.uk),

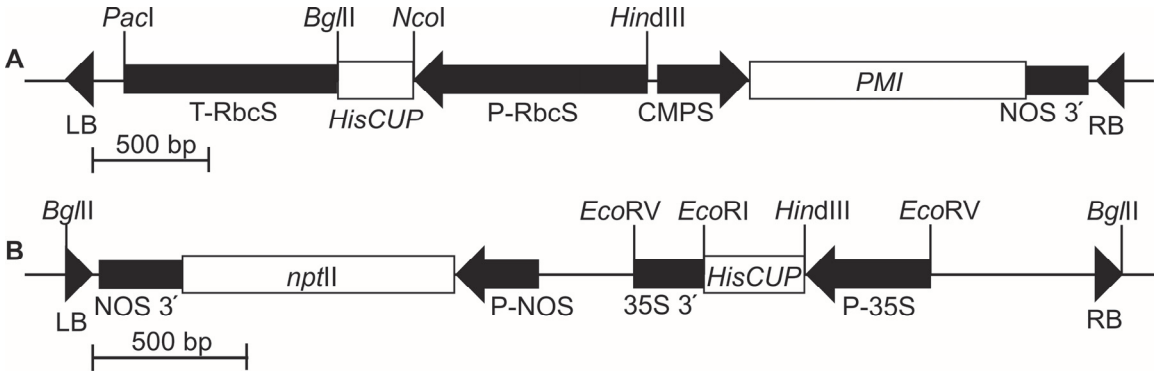


Fig. 1. The T-DNA regions of binary plasmids pNOV2819/RbcS/*HisCUP* (*A*) and pGreen0029/35S/*HisCUP* (*B*). *Pac*I, *Bgl*II, *Nco*I, *Hin*dIII, *Eco*RV, *Eco*RI denote the sites for these restriction enzymes. LB, RB - left and right border region of T-DNA, *HisCUP* - *HisCUP* gene, P-RbcS/T-RbcS - promoter/terminator of Rubisco, CMPS - cestrum yellow leaf curling virus promoter shorter version, *PMI* - phosphomannose isomerase gene, *npt*II - neomycin phosphotransferase II gene, P-NOS - nopaline synthase gene promoter, NOS 3´ - nopaline synthase gene 3´ region, P-35S - cauliflower mosaic virus 35S promoter, 35S 3´ - cauliflower mosaic virus 3´ region.

providing a replicase function for the replication origin of the plasmid pGreen0029 in *A. tumefaciens*. For *HisCUP* gene transient expression in plants, transformed *A. tumefaciens* was used according to the protocol previously described by Nováková *et al.* (2009). Infected leaves were harvested and frozen 3 d later.

Total RNA was isolated from frozen infiltrated leaves using an *RNeasy* plant mini kit (*Qiagen*, Hilden, Germany). After a DNaseI treatment, the RNA concentration was measured using a *NanoPhotometer P330* (*Implen*, Munich, Germany) and only RNA samples with an absorbance ratio $A_{260}/A_{280}$ of approximately 2.0 were used in further analyses. The absence of potential residual genomic DNA contamination was confirmed in control PCR reactions performed with purified RNA before reverse transcription. The RNA (500 ng from each sample) was transcribed into cDNA by *M-MuLV* reverse transcriptase (*NEB*, Ipswich, MA, USA) using an oligo(dT) 23 primer. The mixture for reverse transcription containing 0.8 $mm^3$ of oligo(dT) 23 (0.1 mM), 4 $mm^3$ of dNTP (2.5 mM), 500 ng of the RNA sample, and water up to a volume of 17 $mm^3$ was heated to 65 °C for 4 min. Then, 2 $mm^3$ of an M-MuLV reverse transcriptase reaction buffer (10×), 0.5 $mm^3$ of *M-MuLV* reverse transcriptase (200 000 U $cm^{-3}$), and 0.5 $mm^3$ of an RNase inhibitor (40 000 U $cm^{-3}$) was added, and the reaction mixture was incubated at 42 °C for 1 h. The reaction was terminated by heating at 90 °C for 5 min.

Real-time PCR reactions were carried out using a *CFX96™Real-time* system (*Bio-Rad*, Hercules, CA, USA) in 12 $mm^3$ reaction volumes consisting of gene-specific primers and a *SYBR® Select master mix for CFX* (*Life Technologies*, Foster City, CA, USA). The gene specific primers HisCUP/F and HisCUP/R (described above) for the gene of interest (*HisCUP*) and L25/F (5'-GTT ACA TTC CAC CGA CCT AA-3') and L25/R (5'-GTC AGG AGT CAA CCT CAC AT-3') for the reference *L25* gene (accession No. L18908, Schmidt and Delaney 2010) were used. Each sample was tested in triplicate. The PCR program was as follows: 95 °C for 7 min, 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s. Fluorescence acquisition was measured after each cycle. A melting curve was generated by increasing the temperature from 65 to 95 °C to confirm only one specific product. The baseline and Ct values were automatically calculated by the software *Bio-Rad CFX* manager *(v. 2.0)* (*Bio-Rad*). The relative expression ratio was determined using the software *REST 2009* (*Qiagen*) (Pfaffl *et al.* 2002). Real-time PCR efficiency was determined for each gene by following the procedure: cDNA prepared from 1 500 ng of RNA was diluted 5×, 25×, 125×, and 645×, and the corresponding real-time PCR efficiencies were calculated by the program *REST 2009* according to the equation:
$\text{Efficiency} = 10^{[-1/\text{slope}]} - 1$.

For protein analysis, infiltrated plant tissues (1 g) disintegrated in liquid nitrogen were mixed with 3 $cm^3$ of a buffer containing 50 mM piperazine-N,N′-bis (2-ethanesulfonic acid), 300 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 10 % (v/v) glycerol, pH 7.4. The mixture was further homogenized by a glass homogenizer and centrifuged (18 000 *g*, 4 °C, 1 h). The supernatant (0.2 $cm^3$) was concentrated using a *CentriVap Benchtop* centrifugal vacuum concentrator (*Labconco*, Kansas City, USA) to 0.06 $cm^3$. Proteins in concentrated samples were separated by Tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto a nitrocellulose membrane. A specific mouse *His-tag monoclonal antibody* (dilution 1:2000) (*Merck KGaA*, Darmstadt, Germany) against the histidine tail and *SuperSignal West Femto Maximum Sensitivity Substrate* (including a HRP coated secondary goat anti-mouse antibody, dilution 1:2000) (*Thermo Fisher Scientific*, Rockford, IL, USA) were used for detection of HisCUP MT.

The goal of this work was to prepare different vectors with the *HisCUP* gene suitable for an effective preparation of GM plants and to study the expression of the *HisCUP* gene using a transient expression method. The accumulation of metals in aboveground parts of tobacco with the *HisCUP* gene has been confirmed in previous studies (Macek *et al.* 2002) and it was hypothesized that the gene can function in other plant species, specifically in industrial or fuel crops. The vectors described in this paper vary in the presence of different regulatory sequences and different selection systems. Generally, we have chosen the selection systems of transgenic plants on media containing either mannose or kanamycin and the expression of the transgene under the control of either constitutive or inducible promoters.


Fig. 2. The immunochemical detection of metallothionein in tobacco after using *A. tumefaciens* with pNOV2819/RbcS/*HisCUP* or pGreen0029/35S/*HisCUP*: 1 - a sample of plant tissue treated by *A. tumefaciens* with pNOV2819/RbcS/*HisCUP*, 2 - a sample of plant tissue treated by *A. tumefaciens* with pGreen0029/35S/*HisCUP*, NK - a sample of plant tissue not treated by *A. tumefaciens*.

The vector pNOV2819 contains a gene encoding for phosphomannose isomerase enabling the selection of certain transgenic plant species on a medium with mannose; *e.g.*, rice and flax (He *et al.* 2004, Lamblin *et al.* 2007). This system was chosen to clone the *HisCUP* gene under the inducible Rubisco promoter from *Chrysanthemum morifolium*. This promoter is strongly up-regulated in the presence of light. Outchkourov *et al.* (2003) showed that when comparing β-glucuronidase (GUS) activity in tobacco controlled either by the Rubisco promoter or the CaMV 35S promoter, plants expressing the *gusA* gene under the control of the Rubisco promoter result in an eight-fold higher GUS accumulation than under the CaMV 35S promoter. Using the described genetic components, we have prepared the

pNOV2819/RbcS/*HisCUP* vector (Fig. 1*A*). The second vector was designed with conventional genetic components. These were represented by the pGreen0029 plasmid containing the *npt*II gene (enabling the selection of GM plants that gained the resistance to kanamycin) and by the constitutive CaMV 35S promoter. Using all the described genetic components, we have prepared the vector pGreen0029/35S/*HisCUP* (Fig. 1*B*).

Transient expression in tobacco plants was carried out in order to verify the *HisCUP* gene expression and to compare the activity of the used promoters. The relative expression of the *HisCUP* gene was determined by semi-quantitative real-time PCR. These procedures have been used, *e.g*., by Leckie and Steward (2011) and Visser *et al.* (2012). Eight 5-week-old plants per construct were infected by *A. tumefaciens* bearing either the pNOV2819/RbcS/*HisCUP* or the pGreen0019/35S/*HisCUP* construct and 13 leaves per construct were harvested (each leaf representing one sample). cDNA was prepared from each sample after total RNA isolation and the DNaseI treatment. Thirteen samples for each construct were subjected to the real-time PCR analysis and all the analyses were carried out in triplicates. The expression was normalized to the reference gene encoding for 60S ribosomal protein L23a (an alternative name L25) that is stably expressed (Schmidt and Delaney 2010). The efficiency of real-time PCR, which was calculated from the standard curve by *REST 2009*, was found to be 0.8098 for the *HisCUP* gene and 0.9774 for the *L25* gene. Ct mean values (with standard deviations lower than 0.2) were analysed using the software *REST 2009*. The ratio of target gene is expressed in a sample *versus* a control in comparison to a reference gene. $E_{target}/E_{ref}$ is the real-time PCR efficiency of target/reference gene transcript; $\Delta CP_{target}/\Delta CP_{ref}$ is the CP deviation of control – sample of target/reference gene transcript. This program has been used for a relative gene expression assessment by many other authors, such as Chapuis *et al.* (2011) and Castro-Quezada *et al.* (2013). The plants treated with the pGreen0029/35S/*HisCUP* construct were chosen as control in the analysis and were compared to the plants treated with the pNOV2819/RbcS/*HisCUP* construct. The relative expression of the *HisCUP* gene under the control of the Rubisco promoter was significantly higher (2.416 times; the confidence interval corresponding roughly the same area as the standard error is 0.832 - 6.261; $P = 0.004$) when compared with the CaMV 35S promoter. Outchkourov *et al.* (2003) and Bakhsh *et al.* (2012) also showed that the Rubisco promoter is more efficient than the CaMV 35S promoter. However, the standard error value of the presented data set is relatively high. The high standard error observed in our data set could be caused by either a different number of plant cells infected during the agrobacterial infiltration into leaves and/or different numbers of plasmid copies present in the infected cells. Post-transcriptional gene silencing could also play an important role in the assessment of the *HisCUP* gene expression. Voinnet *et al.* (2003) showed that post-transcriptional gene silencing is the major cause for lack of efficiency of ectopic expression. On the other hand, the entire presented experiment was performed under the standard experimental conditions for both vectors, and the analysis shows statistically significant difference.

The detection of the *HisCUP* gene expression at the protein level was performed by the transient expression. HisCUP MT was isolated, using nickel agarose, from leaves of the plants infected with *A. tumefaciens* bearing one or the other prepared construct. Our experiments show that this purification was ineffective (data not shown). Therefore, the crude concentrated extract from the plant tissue was separated by Tricine SDS PAGE, and proteins were transferred onto a nitrocellulose membrane for a further immunochemical detection. The formation of the HisCUP fused protein was confirmed immunochemically for both prepared constructs (Fig. 2). The transcription and translation of the *HisCUP* gene were confirmed, and therefore, our vectors can be used for a further preparation of GM plants.

Phytoremediation represents a potentially promising method for mitigation of environmental contamination with heavy metals. The enhancement of metal accumulation in plant species with suitable growth properties can eliminate many potential disadvantages of hyperaccumulating plants, such as slow growth rate and biomass production. The two vectors prepared in this work were shown to be useful for the introduction of *HisCUP* gene into plants and for the achievement of effective transgene expression. Our results indicate that the expression of *HisCUP* gene in plants was possible when using both vectors. We also show that using pNOV2819/RbcS/*HisCUP* for *HisCUP* gene expression resulted in higher expression levels than when using pGreen0029/35S/*HisCUP*. A further introduction of *HisCUP* gene into the plant genome using pNOV2819 and RbcS genetic elements can potentially result in successful phytoextraction.

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