Departamento de Bioquímica, Microbiología, Biología Celular y Genética

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Sección BIOLOGÍA

Cloning and structural analysis of the promoter region of cucumber genes encoding Metal Transport Proteins MTP6 and MTP9

Clonación y análisis estructural de la región promotora de los genes de pepino que codifican las proteínas transportadoras de metal MTP6 y MTP9

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Cristina Déniz Henríquez

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Datos Personales

Nº DNI o pasaporte: 51149815-T	Nombre y Apellidos: Cristina Déniz Henríquez
Teléfono: +34 696 477 503	Dirección de correo electrónico: cristinadenizh@gmail.com

SOLICITA la defensa y evaluación del Trabajo Fin de Grado

TÍTULO

Cloning and structural analysis of the promoter region of cucumber genes encoding Metal Transport Proteins MTP6 and MTP9

Autorización para su depósito, defensa y evaluación

D./Dña. Jose Manuel Siverio Expósito	
Profesor/a del Departamento de Bioquímica, Microbiología, Biología Celular y Genética	
y D./Dña. Ana Lancha Bernal	
Profesor/a del Departamento de Bioquímica, Microbiología, Biología Celular y Genética	
autorizan al solicitante a presentar la Memoria del Trabajo Fin de Grado	
Fdo.: Ana Lancha Bernal	Fdo.:

La Laguna, a 8 de julio de 2015

Firma del interesado/a

SR/A. PRESIDENTE DE LA COMISIÓN DE GRADO DE LA FACULTAD DE BIOLOGÍA

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Abstract

Metal Transport Proteins (MTP) group is a universal family of heavy metal transporters who have been determined in archaea, prokaryotes and eukaryotes, and in a serie of plant genomes. They are fundamental membrane divalent cation transporters that transferring metal ions to the cytoplasm's exterior, to the extracellular area and also to several internal compartments. Range of cations recognized to be transferred by proteins of this family consist of: zinc (Zn), manganese (Mn), iron (Fe), cadmium (Cd), cobalt (Co) and nickel (Ni).

This work presents functional analysis of heavy metal transporter MTP6 and MTP9 in plants based on the studies on *CsMTP6* and *CsMTP9* protein from cucumber. MTP6 is a transporter of zinc and manganese and cucumber MTP9 is a transporter of manganese. The gene encoding *CsMTP9* protein was predominantly expressed in roots of cucumber regardless of the stage of plant development, however, a small amount of the CsMTP9 transcript was also observed in leaves.

In this project we review current knowledge of this protein family in cucumber *Cucumis sativus* variety Krak as well as biochemical functions and physiological roles of MTP transporters, comparing with *Cucumis sativus* variety Chinese Long, which has been major help because this genome sequence has already been studied in the past. Phylogenetic analysis suggests that the CDF family has expanded within plants, but a definitive plant CDF family phylogeny has not been constructed. The potential applications of MTP transporters in biofortification efforts are discussed.

Resumen

Las proteínas transportadoras de metales (MTPs) es una familia universal de transportadores de metales pesados que han sido determinados en arqueas, procariotas y eucariotas, y en una serie de los genomas vegetales. Son transportadores de membrana de cationes divalentes fundamentales que transfieren iones metálicos al exterior del citoplasma, a la zona extracelular y también hacia varios compartimentos internos. El rango de cationes reconocidos que pueden ser transferidos por las proteínas de esta familia son: zinc (Zn), manganeso (Mn), hierro (Fe), cadmio (Cd), cobalto (Co) y níquel (Ni).

Este trabajo presenta el análisis funcional de los transportadores de metales pesados MTP6 y MTP9 en las plantas en base a los estudios sobre los genes *CsMTP6* y *CsMTP9* del pepino. MTP6 es un transportador de zinc y manganeso y que MTP9 es un transportador de manganeso. El gen *CsMTP9* que codifica se expresa predominantemente en las raíces de pepino independientemente de la etapa de desarrollo de la planta; sin embargo, también se observó una pequeña cantidad de la transcripción del gen *CsMTP9* en las hojas.

En este proyecto se revisa el conocimiento actual sobre esta familia de proteínas en la especie de pepino *Cucumis sativus* variedad Krak, así como las funciones bioquímicas y funciones fisiológicas de los transportadores, y se realiza una comparación con la variedad Long Chinese, la cual ha sido de importante ayuda debido a que la secuencia de este genoma ya ha sido estudiada en el pasado. El análisis filogenético sugiere que la familia se ha expandido dentro de las plantas, pero su filogenia definitiva no ha podido ser construida. Se discuten las posibles aplicaciones de los transportadores de MTPs en esfuerzos de biofortificación.

1. Introduction

1.1 The importance of heavy metals for living organisms

Heavy metals are a group in terms of chemical of diversified elements with a specific weight of 5 g/cm^3, which in chemical reactions tends to donate electrons (Brewer and Scott, 1983). The group of these elements are included in **metals** (eg. mercury, lead, cadmium, chromium, nickel, copper, zinc, bismuth, manganese, cobalt), **semi-metal** (eg. arsenic, antimony, tellurium) and **non-metals** (selenium) (Duffus, 2002). The release of heavy metals into the natural environment is the result of natural processes such as weathering of rocks stem, volcanic eruptions, evaporation of the oceans, forest fires and soil-forming processes, as well as various human activities. The most significant anthropogenic sources of environmental pollution with heavy metals include:

1. Metallurgical and chemical industry
2. Mining and metallurgy non-ferrous metals
3. Landfilling of waste
4. Use of high doses of contaminated mineral fertilizers (mainly phosphorus) and pesticides, as well as intensive development of communication, derived from these sources.

Heavy metals in the environment contaminate soil, water, air and directly or through plants they invade the body of animals and humans. The increasing concentration of heavy metals in cultivated fields is, therefore, a particular threat to the environment and human health. Detailed analysis of metals soil contamination showed that about 100,000 hectares of fields in Europe and the USA should be excluded from food production, due to excessive pollution of heavy metals (Lebeau et al., 2008). Due to the degree of danger, heavy metals were divided into four groups:

- **Group I** → includes metals with a very high degree of potential hazards. For example: Cd, Hg, Pb, Cu, Zn.
- **Group II** → contains metals with a high degree of potential hazards. For example: Mo, Mn, Fe.
- **Group III** → contains metals that occur with an average degree of potential hazards. For example: Ni and Co.
- **Group IV** → included metals with a low degree of potential hazards. For example: Sr, Zr.

In organisms, there are an extensive network of homeostatic mechanisms, aiming at maintaining the balance of intracellular concentrations of heavy metals, necessary for the proper functioning of cells (iron, zinc, copper, manganese, cobalt), cell metabolism and protection from the toxic effects of ballast metals (lead, cadmium, mercury , arsenic, antimony, barium).

Heavy metals necessary for the life of organisms is ranked among the micronutrients. These are the elements, which occur in many enzymes active centers or transcription factors, which determines the correct course of most of the key metabolic reactions. Each of micronutrients meets specific physiological functions and therefore can not be replaced by another metal:

- Zinc is a cofactor for more than 300 enzymes and 2000 transcription factors. It is involved in the synthesis and metabolize proteins, maintenance of membrane integrity and reproduction, as well as in the metabolism of fats and carbohydrates. At the plant it is indispensable for the production of auxin and proper course of nitrogen metabolism. (Marschner, 1995; Barker and Pilbeam, 2007; Prasad, 2012).
- Manganese activates the enzymes involved among others in DNA synthesis, deactivation of reactive oxygen species generated during oxidative stress, metabolism of sugars; also plays a key role in the process of photosynthesis when water oxidation in photosystem II (Marschner, 1995; Crowley et al., 2000; Merchant and Sawaya, 2005; Williams and Pittman, 2010).
- Iron is necessary for the proper course of redox reactions in chloroplasts and mitochondria, participates in the synthesis of chlorophyll and DNA replication, as well as in the process of assimilation of atmospheric nitrogen (Marschner, 1995; Briat et al., 2007).

As deficiency of these metals is limiting for growth, plants have developed specific acquisition and transport mechanisms for each of them. In recent years, much more it emphasizes the importance of zinc in the life of living organisms. In addition to iron, zinc is the second metal whose deficiency in organisms is quite common in countries with high levels of poverty, resulting in serious disturbances in the development of animals and humans. In 2008, the Copenhagen project participants (called *Copenhagen Consensus*) aimed at finding solutions to the biggest problems of mankind on the basis of scientific and economic analysis found that zinc deficiency in the diet, in addition to vitamin A deficiency is a priority

issue for global and, the elimination of this problem will have an immediate positive effect on the population of developing countries.

1.2 Plants as an important source of micronutrients

The first link in the chain lively circulation of matter, called ‘the food chain’, plants are downloading from the soil elements and chemical compounds needed to build living cells. Depending on the composition of the soil solution, plants absorb less (under deficit) or more (in terms of excess) elements from the substrate, in connection with this in accumulation in their tissues both compounds necessary for life, as well as toxic compounds, including heavy metals. Plants are the main source of metals in animal diets. In turn crops and farm animals meat is a key source of micronutrients such as Zn, Cu, Fe and Mn in the diet of man. As a result, the level of metals accumulation in plant tissues determines the availability of these elements for the organisms of animals and humans. Thus, inadequate or excess intake of metals from the soil by plants will influence the deficiencies or toxic surplus of these elements in the diet of heterotrophic organisms, leading to their serious illness and even death. There are many factors that have an impact on the collection of metal ions contained in the soil for plants: Physical and chemical properties of the soil determines the aqueous solubility metal salts, mobility and availability of free cations in the soil solution, the content of organic matter in the soil and the degree of its hydration have an effect on the ability of the soil to cation exchange, and thus the availability of heavy metals (Balasoiu et al., 2001; Tom-Petersen et al., 2004). The pH of the soil determines what form the metal is present in the soil solution high pH favors the formation of insoluble metal complexes, which greatly decreases their availability, and in conditions of low pH metals are generally present as free ions, readily taken up by plants (Sandrin et al ., 2007).

Similarly, also affect the redox potential of the soil to collect metals from the environment: metals under oxidative conditions occur in the form of free ions, which exhibits greater solubility in water and are more available to plants (Olaniran et al., 2013).

1.3 Metal Transport Protein (MTPs) family

As well we have said, metals can be harmful when in excess. Mn and Fe toxicity are two of the main limiting factors for agriculture in acid soils, which cover 30% of the planet, whereas Zn toxicity is observed in contaminated soils around mining sites (von Uexküll and Mutert, 1995; Krämer, 2010). Excess of both Zn and Mn impairs growth and leads to chlorosis, competing with other ions for binding sites (e.g., leading to Fe deficiency).

To avoid cellular damage, heavy metals are generally chelated by low molecular weight compounds, sequestered into organelles or expelled to the extracellular space by specific transporters. One class of metal transporters involved in these functions is the Cation Diffusion Facilitator (CDF) family, also known as **Metal Tolerance Proteins** (MTPs).

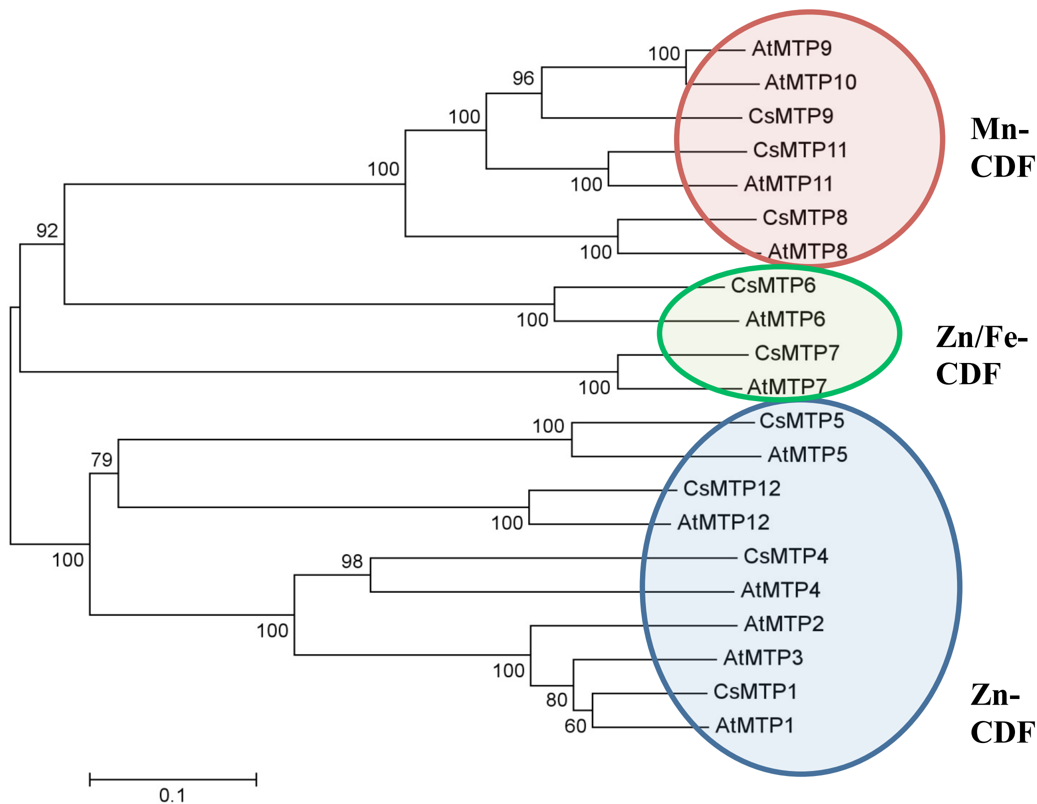

Figure 1. There are three subfamilies of Metal Transport Proteins (MTP) named after the substrate specifity of their members: Zn-CDF (zinc transporters, MTP1-5 and MTP12), Fe/Zn-CDF (zinc/iron transporters, MTP6-7) and Mn-CDF (manganese transporters, MTP8-11). Until now, only the function of MTP1 and MTP3 has been established: both transporters reside at tonoplast or plasma membrane and participate in zinc transport into vacuoles or cell wall, respectively.

1.4 Cucumber *Cucumis sativus* variety Krak

The botanical family Cucurbitaceae, commonly known as cucurbits and gourds, includes many economically important cultivated plants, such as cucumber (*Cucumis sativus* L.), melon (*C. melo* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), squash, and pumpkin (Cucurbita spp). Agricultural production of cucurbits utilizes nine million hectares of land and yields 184 million tons of vegetables, fruits, and seeds annually (http://faostat.fao.org). The cucurbit family also displays a rich diversity of sex expression, and the cucumber has served as a primary model system for sex determination studies.

Furthermore, the cucurbits are model plants for the study of vascular biology, as both xylem and phloem sap can readily be collected for studies on long-distance signaling events that function in the integration of physiological and developmental processes at the whole-plant level. Despite their agricultural and biological importance, cucurbits are a group of under-investigated or 'orphan' crops that have few genetic and genomic resources.

Cucumber (*Cucumis sativus*) is a universally cultivated plant in the gourd family, Cucurbitaceae. It is a creeping vine that carry cylindrical fruits whic are used as comestible vegetables. There are three principal varieties of cucumber: slicing, pickling, and burpless. Within these varieties, there are several different cultivars which have emerged. The cucumber is originally from East Indies, but now grows on most continents. It has seven pairs of chromosomes and a haploid genome of 367 Mb, which is smaller than other species in Cucurbitaceae family. As the first sequenced vegetable crop, cucumber genome will provide an invaluable new resource for biological research and breeding of cucurbits. To better manage the cucumber genome data and facilitate public academic users to access the genome data and related information, it has been developed the Cucumber Genome Database.

2. Aim

The aim of this work can be summarized in two key points:

- The amplification of promoter sequences of cucumber variety Krak genes *CsMTP6* and *CsMTP9*
- Identification of regulatory *cis*-elements promoter sequences of *CsMTP6* and *CsMTP9*

3. Material and methods

3.1 Bioinformatic analysis

3.1.1 Bioinformatics algorithms used in progress

The program that was used to analyze the results of sequencing the plasmids with the genes of cucumber is named **Multalin** (Corpet, 1988), for alignment sequenced amplicons generated sequences of genes.

3.1.2 *Database of which was extracted genomic contigs containing genes MTP or ready sequences of genes encoding proteins vegetable MTP*

PlantCARE is a database of plant *cis*-acting regulatory elements, enhancers and repressors. Regulatory elements are represented by positional matrices, consensus sequences and individual sites on particular promoter sequences. Data about the transcription sites are extracted mainly from the literature, supplemented with an increasing number of in silico predicted data. Apart from a general description for specific transcription factor sites, levels of confidence for the experimental evidence, functional information and the position on the promoter are given as well. New features have been implemented to search for plant cis-acting regulatory elements in a query sequence. Furthermore, links are now provided to a new clustering and motif search method to investigate clusters of co-expressed genes. New regulatory elements can be sent automatically and will be added to the database after curation.

3.2 Biological material

In the present study were used in all experiments:

- *Plant material*: seedlings and plants cucumber ground. (*Cucumis sativus* L. variety Krak)
- *Microbial material*: modified strains of *Escherichia coli* (genotype TOP10, F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG)

3.2.1 *Cucumber plants growing conditions*

Ingredients	Final concentration
KNO_3	1,7 mM
$Ca(NO_3)_2$	1,7 mM
$MgSO_4$	0,33 mM
KH_2PO_4	0,33 mM
Microelements:	
$Fe(C_6H_5O_7) \cdot 6H_2O$	25 µM
$MnSO_4$ x $5H_2O$	3 µM
H_3BO_3	1,7 µM
$CuSO_4$ x $5H_2O$	0,003 µM
$ZnSO_4$ x $7H_2O$	3 µM
Na_2MoO_4 x $2H_2O$	0,017 µM

Figure 2. Composition of the medium used in hydroponics cucumbers. Media pH value was adjusted to 6.0 (no heavy metals) or 5.5 (when to mush introduced additional portions of heavy metals). Media was changed at least 2 times per week.

Cucumber cultivation was carried out in hydroponic systems. Seeds of plants were sown in trays lined with moistened filter paper lined, in which germinated in the dark for 48 hours at 25 °C. Then germinated seeds were planted on the jars containing a medium having the previous composition. pH of the medium was adjusted to 6,0. Plants grown indoors greenhouse room, under continuous illumination (16 hours a day and 8 hours overnight) and temperature (25 °C during the day and 22 °C overnight). The medium was exchanged twice a week.

The plants, which have been collected the material to examine the expression of organ, grown for 2 weeks. Roots, cotyledons, hypocotyles, leaves and petioles were collected for DNA preparation and immediately frozen in N_2 liquid. After this, they were ground in a mortar with N_2 liquid too, in order to homogenized the material, and stored at – 80 ºC.

3.2.2 Cell culture conditions of Escherichia Coli bacteria

The research was conducted with a chemically competent bacteria E. Coli TOP10, of genotype F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG (Invitrogen).

Bacterial cultures were grown on standard media LB (Luria-Bertuli) prepared on the basis of doubly distilled water, with a given composition of: 1 % (w/v) tryptone, 0,5 % (w/v) yeast extract and 1 % (w/v) NaCl. Its pH was adjusted to 7,0 with 1M NaOH. Solid growth medium was obtained adding to the media 1,5% of liquid agar at a final concentration. After transformation the bacteria were grown for 1-1,5 hours at SOC medium (called *Super Optimal Catabolite* repression) of the composition: 2 % (w/v) tryptone, 0,5 % (w/v) yeast extract, 0,05 % (w/v) NaCl, 2,5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM sterile glucose. The medium was autoclaved at 121ºC at a pressure of 1 MPa for 20 minutes. The selections of transformants was performed on LB media enriched in antibiotic kanamycin with a final concentration of 100 ug/ml.

Bacterial cultures were performed at 37°C in the dark. Bacteria were stored as a suspension in 15% glycerol solution at the temperatura of -80ºC (long-term storage) or in Petri dishes with the ground selection in the refrigerator (storage of short-term).

3.3 Molecular biology methods used in the course of work

3.3.1 *DNA isolation from cucumbers*

We use a CTAB DNA extraction protocole. We use the following solutions:

- CTAB buffer: 2% CTAB (Hexadecyltrimethylammoniumbromide), 100 mM Tris-HCl pH 8,0, 1,4 M NaCl, 20 mM EDTA, 0.5 % beta-merkaptoetanol. Prior to starting extraction, we added β-mercaptoethanol.
- Isoporpanol
- 70% etanol
- Chloroform
- TE buffer/sterile water.

We grinded tissue in liquid nitrogen with pestles, keeping tissue frozen the entire time. Then we transferred 100 mg of frozen tissue to fresh eppendorf tube 1,5ml, we added 500 uL of CTAB buffer and mix the tubes. After that we incubated at 65 °C for at least 30 min, mixing the tubes sometimes. We removed samples from waterbath and IN FUME HOOD, added 300µl of chloroform and mixed by gently shaking tubes. We centrifuged for 10 minutes at 14,000 g. We transfer the aqueous phase (top layer) into the new labeled tube. We stimated the volume of the aqueous phase and added equal volumes of cold isopropanol. We mixed by inverting tubes 10 times and incubated on ice for 10 minutes and centrifuged for 10 minutes at 14,000 g. After that, we discard supernatant, we added 1000 µl 70 % EtOH, we inverted tubes 5-10 times and we incubated the samples for 1 hour at room temperature.

Now we centrifuged the samples for 10 minutes at 14,000 g, we discarded supernatant and we inverted tubes on a clean kimwipe, allowing to dry for 10-15 minutes upside down, or until pellet looks dry. Finally, we hydrated pellets with 50µl TE/sterile water, allowing to resuspend overnight at room temperature. The DNA should be stored in the refrigerator the next day.

3.3.2 Quantitative and qualitative determination of nucleic acids spectrophotometric method

The quantity and purity of nucleic acids isolated were checked by spectrophotometry using Nanodrop, Thermo Fisher Scientific. Measuring the absorbance of samples was made at three wavelengths: 230 nm, 260nm and 280nm.

3.3.3 PCR reaction

To analyze the expression of the organ, as the template cDNA transcribed from RNA isolated from various plant organs. 1µl cDNA was introduced to the reaction of the following composition: 5 µl of sterile deionized water ,10µl polymerase buffer Marathon (2x conc with 4 mM $MgCl_2$), 1 µl Marathon polymerase (1U / µl), 1 µl of 10 mM dNTPs, 1 µl of forward primer and 1 µl of reverse primer.

Reactions were carried out in a thermocycler under the conditions shown in the table. At the same time, the same reference gene was amplified cDNA encoding elongation factor.

Stage	Temperature	Time	Repetition
Prior denaturation	94ºC	10 min	1
Amplification			25-30
• **Denaturation**	94ºC	30 sec	
• **Annealing**	58ºC	30 sec	
• **Elongation**	68ºC	1 min	
Final elongation 68ºC 10 min		1	

Figure 6. PCR reaction conditions used for the analysis of gene expression *CsMTP6* and *CsMTP9* in various organs of cucumbers.

3.3.4 Electrophoresis of nucleic acids

PCR products nucleic acids purified from agarose gel were separated by electrophoresis on 1,5 % agarose gel in TBE buffer once concentrated (89mm Tris Base, 89 mm boric acid 2 mM EDTA, pH 8,0). Attempts was stirred with loading buffer SB (10 mM TE buffer, pH 8, 30% glycerol, 0.35% bromophenol blue) in a ratio of 5:1 in the case of PCR and then applied to the wells in the gel. To check the size of the resulting products on the gel was applied also 3µl 1 kb DNA marker GeneRuler Plus (0,1µg/L, Fermentas). Electrophoresis of PCR products was performed at a voltage of 100 mV in about 30 minutes. After electrophoresis, the gel was transferred to an aqueous solution of ethidium bromide (10µg / ml) for 15 min and then rinsed in water for 5-10 min. Stained PCR products were viewed and recorded on the camcorder UV light.

3.3.5 *Purification of PCR products from agarose gel*

After registration, nucleic acids results in the camera UV transilluminator gel was transferred to UV light and excised with a scalpel amplicons were transferred for further research. For the extraction of DNA fragments from the gel, a kit GeneJET Gel Extraction Kit (Thermo Scientific) was used.

Excised gel fragments were placed in Eppendorf tubes (1,5 ml) into which the solution of nucleic acid-binding membrane silica (called. *Binding Buffer*) in a ratio of 100:l of a solution for every 100 mg of gel. Samples were incubated in a thermoblock at 65 ° C until complete dissolution of the gel every few minutes, stirring them by inversion, and finally briefly shaking. The resulting solution with pipette transferred to columns with a bed of silica and centrifuged at 14,000 g for 1 minute, after which the filtered solution was discarded. Then the columns were washed twice by applying 700 µl wash solution (called. *Washing Buffer)* on silica deposits and whirling attempts at 14000g for 1 minute. After washing, samples were centrifuged again under the same conditions to remove the remaining wash solution. Thus prepared columns were transferred to a new Eppendorf tubes. The agent deposits of silica was applied 35 µl of elution buffer (called. *Eluttion Buffer*), and the mixture was centrifuged at 14,000 g for 2 minutes to wash acids related to the stanchion to the tube.

In order to check the DNA of the resulting samples was carried out a control agarose gel electrophoresis by applying the gel 5 µl of the extract with the addition of 5 µl of loading buffer and also is the concentration of the extract on the NanoDrop spectrophotometer.

3.4 Primers used at work

Below shows the sequences of the primers used in PCR reactions carried out in the course of work:

- M13 Forward primer 5′-GTAAAACGACGGCCAG-3′
- M13 Reverse primer 5′-CAGGAAACAGCTATGAC-3′
- P6 Forward primer → GGGGACAAGTTTGTACAAAAAAGCAGGCTTC AAC CAG CAA CAA CAG CAG AG
- P6 Reverse primer → GGGGACCACTTTGTACAAGAAAGCTGGGTC TAT GGG ATT GAG ACG GTG GAA T

3.5 PCR cloning into a pDONRTM vector

3.5.1 pDONRTM bacterial vector

pDONRTM vectors are designed to generate attL-flanked entry clones containing the gene of interest following recombination with an attB expression clone or an attB PCR product. After creating an entry clone, the gene of interest may then be easily shuttled into a large selection of expression vectors using the LR recombination reaction.

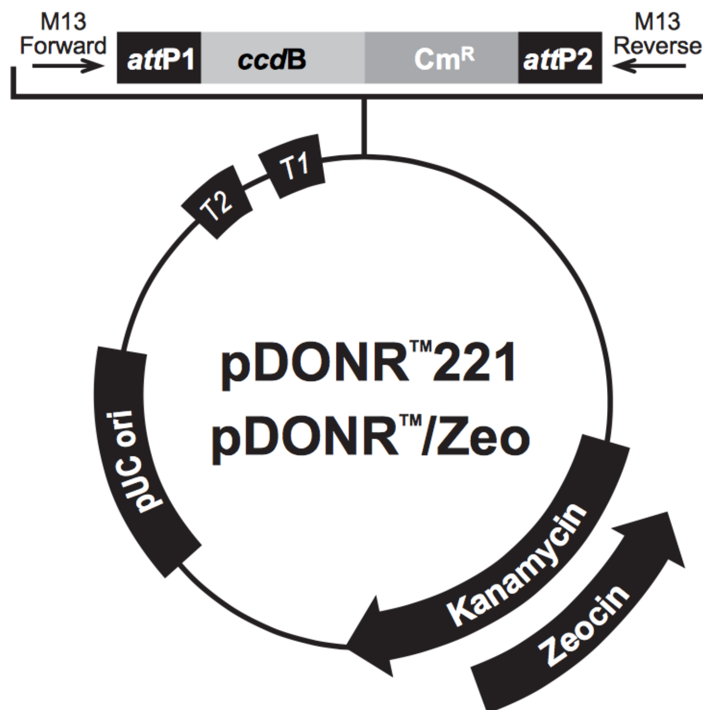

Figure 3. The pDONRTM vector that we use, called pDONR™221, contain the following elements: - rrnB T1 and T2 transcription terminators for protection of the cloned gene from expression by vector-encoded promoters. -M13 forward (−20) and M13 reverse priming sites for sequencing of the insert. -attP1 and attP2, recombination sites for recombinational cloning of the gene from a PCR product. -ccdB gene located between the two attP sites for negative selection. -Chloramphenicol resistance gene located between the two attP sites for counterselection. -Kanamycin resistance gene for selection in *E. Coli*. -pUC origin for replication and maintenance of the plasmid in *E. coli.*

3.5.2 BP reaction

We have to add the following components to a 1.5 ml microcentrifugate tubes at room temperature and mix: attB-PCR product (=10 ng/µl; final amount ~15-150 ng) 1-7 µl, PDONR vector (150 ng/µl) 1 µl, TE buffer, pH 8.0 to 8 µl.

After that it is needed thaw on ice the BP Clonase II enzyme mix for about 2 minutes. Vortex the BP Clonase II enzyme mix briefly twice (2 seconds each time). To each sample and positive control, add 2 µl of BP Clonase II enzyme mix to the reaction and mix well by vortexing briefly twice (not add BP Clonase II enzyme mix to the negative control). Then, microcentrifuge briefly and return BP Clonase II enzyme mix to -20 °C or -80 °C storage immediately after use. At the end, reactions must be incubate at 25 °C for 1 hour. Finally, add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37 °C for 10 minutes.

3.5.3 Transformation of chemically competent bacteria

After performing the BP recombination reaction, we must transform competent *E. Coli* bacteria and select for entry clones using kanamycin antibiotic. We will need 2 LB plates containing kanamycin for each transformation, which have to be pre-warm at 37 ºC for 30 minutes. We take a tube that containing 50 ml of suspension using a pipette bacterin automatically placing 2 µl ligation mixture, then the mixture was gently stirred and incubated on ice for 20 minutes. After this time, the mixture was transferred to a water bath at 42 °C for 45 seconds, subjecting the bacterial cells were heat shocked and then quickly placed on ice again. After two minutes, the test tube was added 250 µl of SOC medium and shaken at 150 rpm at 37 °C for 1 hour. The transformed cells were plated on Petri dishes with selective LB medium supplemented with appropriate antibiotics. The plates were then incubated in an incubator at 37 °C for 24-48 hours. The BP reaction should give > 1500 colonies if the entire BP reaction is transformed and plated. True entry clones should be kanamycin-resistant.

3.5.4 Analysis of entry clones: Bacterial PCR

We need to analyze the entry clones by restriction analysis to confirm the presence and correct orientation of the insert. We picked 5 colonies and cultured them overnight in LB medium containing kanamycin antibiotic.

Bacterial PCR was performed on the DNA template single colonies of bacteria in a mixture of the following composition: 10µl of 2x concentrated polymerase buffer Marathon (containing 4 mM $MgCl_2$), 0,5µl Marathon polymerase (1 U/ul), 0,5 µl mixture of 10 mM dNTP, 0,5µl 10M forward primer, 0,5 µl 10M reverse primer and 8 µl of an aqueous suspension of bacterial colonies. The presence of inserts in the plasmids transformed bacteria was verified by PCR reaction using these specific primers for the analyzed vector.

Stage	Temperature	Time	Repetition
Prior denaturation	94ºC	10 min	1
Amplification			30
• **Denaturation**	94ºC	30 sec	
• **Annealing**	58ºC	30 sec	
• **Elongation**	68ºC	1 min	
Final elongation 68ºC 10 min		1	

Figure 5. Terms of bacterial PCR with primers specific for the test vector and bacterial colonies as source of template DNA.

The resulting PCR products were subjected to electrophoretic separation on a 1 % agarose gel. Colonies in which the presence of the plasmid was confirmed were grown in 3 ml LB liquid medium supplemented with appropriate antibiotics for 16 hours. With this prepared of liquid bacterial cultures plasmids were isolated for sequencing and transformation of yeast and protoplasted.

3.5.5 Isolation of plasmids

Isolation plasmids of liquid bacterial cultures was performed using GeneJET Plasmid Miniprep Kit (Thermo Scientific), 3 ml of bacterial suspension was centrifuged at 14,000 g for 5 minutes. The supernatant was discarded and the resulting pellets were thoroughly resuspended in 250µl of a suspension (called *Resuspension Solution*). TEST fed with 250µl lysis solution (called *Lysis Solution*) and the mixture was gently stirred by inverting 4-6 times. Subsequently adding 350ul of the neutralizing solution (called *Neutralization Solution*) and the mixture was gently stirred by inverting 4-6 times. This sample preparation was centrifuged at 14,000 g for 5 minutes. The resulting supernatant was transferred to a small column of silica deposits and centrifuged at 14,000 g for 1 minute. The filtered solution was discarded and material tied in small columns were washed twice with 500µl wash solution (called *Wash Solution*), each time swirling attempts at 14,000 g for 1 minute. Then columns were transferred to new Eppendorf tubes (1,5 ml) and the surface of the layer of silica was applied to the 50µl of elution buffer (called *Elution Buffer*). Attempts were incubated at room temperature for 2 minutes, then centrifuged at 14,000 g for 3 minutes.

Concentration and purity of isolated plasmid was determined using a NanoDrop spectrophotometer. The presence of the insert in the resulting plasmids were verified arranging for sequencing samples.

4. Results

4.1 Amplification of promoter sequences of cucumber variety Krak genes *CsMTP6* and *CsMTP9*

4.1.1. MTP6 promoter

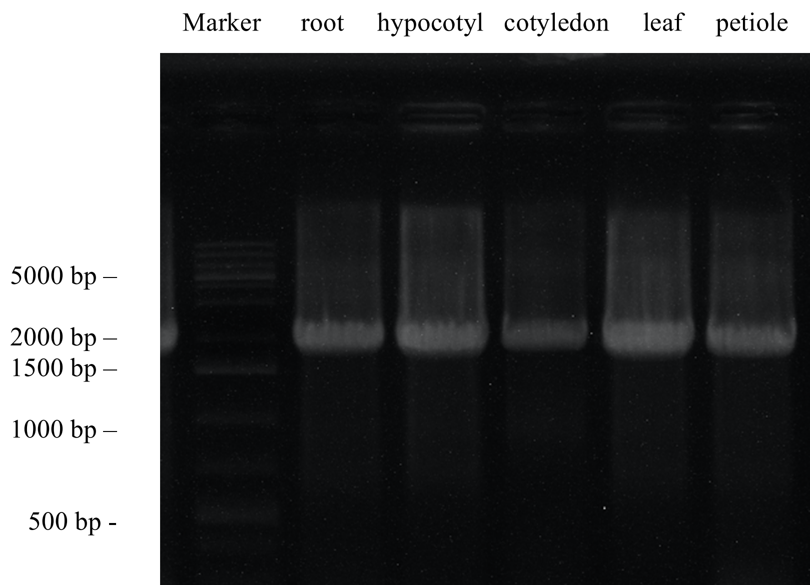

Figure 7. *Amplification of MTP6 promoter. PCR reaction was performed with DNA isolated from the roots, hypocotyles, cotyledons, petioles and leaves of 2 week-old cucumber plants.*

```
>promoterMTP6Krak
AACCAGCAACAACAGCAGAGACAAAGGAGTCCTTATGGATATGGAGAGCAAAACAGATATGGAAACAACAACAGAAGATGGCGTGATAGAGAATAT
TGAAACTGATGAAATTGTTGTTCGAGTAAAAGATGATGAGACTTTCCCTACTCATAGAGAAGAAGCTGATGCTTTTGGTTTTGGGCGTTGAAAATG
GGTTTAAAAAGCATTGAATAAGAGCTTCTCCTTGGCGTTCTTCTGTATTTTGCAAGTTTTTTCTTCCTATACTTTCAAAGCCCCTTGAAGAATGAAA
ATAACACAAATTCACAACAGCTGACTGTTACCAGAAGTATGCTCCTTTTTTCTAAAAAAAAAAAATTAAAAGTTAAAAATAGGGGCTTTGGCTTTT
GACCTGTAAAATTTAGTTCCATGAGCTTGGGTTTGTAGGGGAGGGGGGGGATTTTATTTTTCAATATCAATTATTATTAGTGTTACCTTCTTGTTT
TTGAAAGTGTGTTGATGAGTTCATAGATCTTCCAATTTTCAATCTAAATTTGAATGAGGCTTCAGCAATTTAGTACTTTCCAGTTCCTAACATGAA
ATCTTTTTATAGATGTTGAATTTCATCCTCAAATGGATATATTTATATTGCCAAATAATTAAAAAGATATCTTAAAAAATAAAGGGGAAAAAAAAG
CCTATGTTTATTTTATTTTCTACTTTTGGAAAGAAACACAACAAAGATCAGACATCAACGTAACATCTTCCCAGCATCCATCTCAGGCTCAACAAGA
CTAGATAGCCAAAGGCGGAAACAGAAAAGCATCGATCCAAGAACAAGGTGACAAATTCGAAAGAGCATAGTTTGATCAAGACGAAAAGCATCGATT
CAAAAACAAAACAACAAAATCCAAAGACCACGATTGGCCTAATTTGAGAATTGATTGGTCAAATTTCAAAAGTGTGTCGTGAATTTATATATATAT
ATATATATATATATATATGATGTGATATATGTGTATATATTTTCCAATTAGATCTATTCACCATTTTAAATAAAATACATCCATACATCTTTTAAA
TGTAACAATCAACTTTTTTTTTTCAATTGACATATGTTCTTTCAGTTTACATTTTTTTGGGAGAGATCTCTGAGTTTGCATGATATTTGTTGGGCA
TAAAATTAAAAGTGTCTAAACGTTCTAAACATAATATTGAAATTTGTAAGAACGAATCAAAATTGAAATTTGTAAAGATGATTAGAGTGTAATGC
TATATATAATCTTTCTTCAATTTTAATTTATTTAATTTCAGAATTTTAATCTTAATTTCTATACTTTATTTGAAAATGATGGAAAATAACAAAATT
ACTGAAATATTTACGAAATATAATCGATTTTTAAATTTTATCACTAACCAACCTTCTAATTAACAATATAAATATATTGATATCAATATATACAAT
AATAAAAGTATAAATATAATTTGAAAAATTAATATTTTTGTCTAAATTTTATATTTCAAAATAGTCATAGCTAATAACTAAATAGAATATAGTTC
AATTAATATATAGAGTTTTTCAAATAATAGGAAAAAAAAATATTTATACTGCATACAAAAATTCATTACAAATCCATTTTTATTTTTGAAATTTTT
TCTAAAGTTTTCGTTAAAATTATAATAACTAAAAGTATAGATGCCAAAATAATAATAAAAATAATAATAATAATAATAATAAAAAAAAAAAAAAA
AAAAGGAAACAGCATGATCGGTGGAGAGACGAAGGTTCTGAACAGAGGAACAAGATTCCATTTTTCTCACAAATCTGTACAGAATCGGTGCTTCTAC
TCTGTTCCTTTTACTCTTTGTTTCATCTCTTCAACTTCCATTCACCAATTTGTGAAATTAAACATCATTTTTCACAATCCTAAAGCTTCTGATCATCA
AAATCCATCTCTTCCCCACCAAAAACTCCATTAAAATGGGATACAGATTCCACCGTCTCAATCCCATA
```

Figure 8. Sequencing of MTP6 promoter. The part of sequence coding protein is underline and the the start codon is shown in red colour.

The PCR product cucumber variety Krak that we obtained was **1990 bp**, but initially the promoter size was *1957 pb* for variety Krak and *1947 pb* for variety Chinese Long

4.1.2 MTP9 promoter

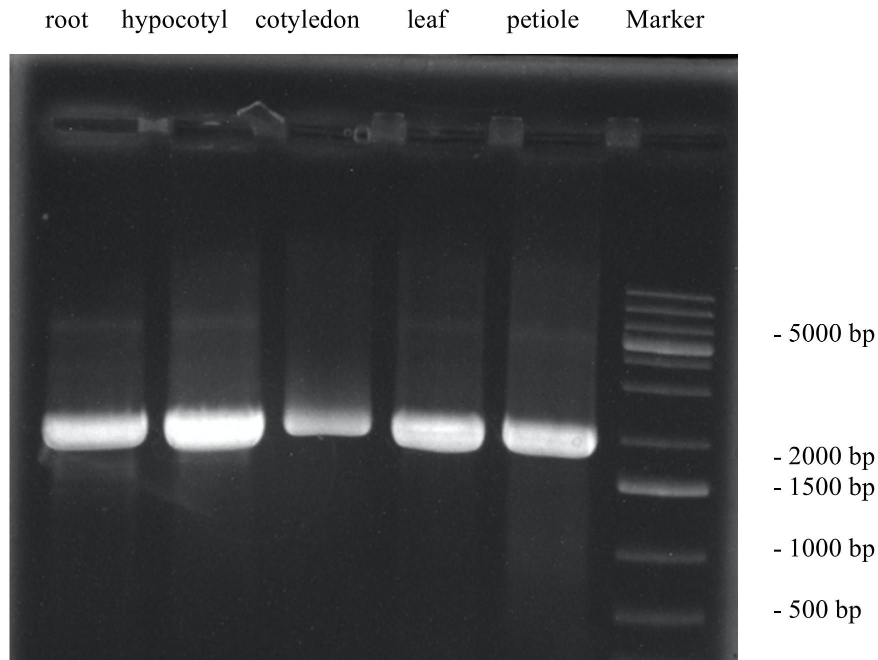

Figure 9. Amplification of MTP9 promoter. PCR reaction was performed with DNA isolated from the roots, hypocotyles, cotyledons, petioles and leaves of 2 week-old cucumber plants.

>promoterMTP9Krak

GGGCACACACGTCACATATCATCATATTTTTTATAAAAGTTTCAGTTGGTGAATTAGGTTCAACAACTCCTCTACATCAATATAAAACAAAGAGAAA
TTGCTTTAAAATATATAAGTGCTTTTCCTTAGCTAATTTAAGAATTAAGGGGATGCTTGGACTCCTAATTTTTAAAAGGGAGTGGAGTAAACTATC
ATCTTCATGATTGCGACTTTAATTATAATAATAGTAGGCGTTTCCAATTAGTATCAACAACTATATTTCTATTTCTATTATTTGGACTATTTTCCTTC
TATATTAAAATAGTTCAGTACATACTATTTTTAACTTATACTAAAATGGACTATTATAATCCTACAGTAAGCAACTAAGCACTACAAACATTCCTTAT
AATATTCAATTCAATTTGATAAAATTTTGATATTCTTTTCTTTAACCCTTTGTTTGGATTCTAGAAAGTTGAAGAAATAATTTTAAATTTTATGAT
ATGAAATTTTAGAAATGATATGAAATTATTTATTTTGTTTGAAACCAGAAAGATTTGAAATAAATAAAATTTCTATGTTTGGATGATAATTAAAA
AGAAAAATAAAGTTATGAATTTACAAATAGAGACTGTTATCTGCAAATGTGTTAAAAGTGAAAATTAGAAAATGAAAAGATGAATTATGTGATTT
TCTTTAAAATTCTGCAAAATCTGACCATTTTGGTCAAGAAAATTTAAGTAAAAACATGATTTAAAATCATTCTAGACTTTAAAATCATTCTCTTTT
TGCTATAACAAACAATAGAATTCATTTAAAATTTCTCAAAATCTAGAGGTTCTAGAGAGAACATAGGTGAGATTTAATTATTTGTAGGACTGAAAA
ATGTGTCCAAATTGTTATTGAATCGCAACTTATCACTAAATGATATTATTGATAGACAATGACATTTTGCTATACTAAAAATATTTTCAAAAGTTTT
ACTATTTCAAACGACTAGCCACTCAAATTATCAACTTTGCACGAGTCACATTGTAACTTCGATTTTATACAACTTTTAAAAGATTTATTTTGTGGC
AGGGATATTTCAAACTTATAAGTAAAACTAAACGAGAAAAGGATTTAGTTGTCTATTTTTAGAAAATAGAGAACAAGAAATATAGTAATTATCAGT
GAATACAGATTCAGTTTTTATTAAAAGGAAAACAAGGACAAAAAAAAAAATGATAATCAAACATATAATTTAGAAAAAAACGTAAGATAAAGTTAA
TTTAATGGGGACAGAATGTAATGATATGAAACACAAACTTCCGAAAAGAACATAAGTACTTCAACCTTGGTTTTACCCCCAACAATGCTCCAACCAT
AAAAATAAAATAATCATTCTTCATAATTCTATCCCCAAAACACCTCTCACACCAGATTTCCCTCAATATAATAAAACATCAATGACCAAACAATCTTC
AAAAAGACTTAAAAACAATCATCATAAACCATAGTTGAAAAAAAAAACATAAAAGATTTTGTTATACTTGTAGGAATGCACGTAAGATCCTACAAA
GCAATTCCCATACTTCAATATTATTCAAACCAAACCTTTAACTTTCCCCAGCACTCATTCAAAATGTCAAATCACCCCTTCCCTTAAATATCCAACCC
TTTCCATGCCATTGTTTACTGAATCCACTTTTCTCTTATTATATTCATCACGTCCATTTTAGTCTTCTTTCCCTTCTTTATAATGATCTCTTTGGTTT
TAAGAAACAGAAAATCGCTTCCCTTTTAACTAGAATCTTAGTTGAGTTCACCATAAAAATGGCAGATAATCCTAGAACAGATTCCTTCAGAACAGA
GCTTCTGTCACCGGAGGGCGTGGCAGCTGGGACAGATGGAACGGTGAGGAAGGTGCCCTCGTGGCGACTCAAT

Figure 9. Sequencing of MTP9 promoter. The part of sequence coding protein is underline and the the start codon is shown in red colour.

The PCR product cucumber variety Krak that we obtained was **1907 bp**, but initially the promoter size was *1796 pb* for variety Krak and *1795 pb* for variety Chinese Long.

4.2 Comparasion of the *CsMTP6* and *CsMTP9* sequences from cucumbers Chinese long and Krak

Nucleotide sequence of cucumber genes, called *CsMTP6* and *CsMTP9,* were previously generated by an algorithm called MultAlin and primers were complementary to the beginning and end of the gene sequence. These primers are used in a PCR reaction to amplify the complete coding sequence of the genes. Sequencing products obtained revealed some differences compared with the expected sequence generated by the algorithm.

4.2.1 MTP6 promoter

Figure 9. Comparison of the generated and obtained MTP6 promoter sequence. The predicted gene sequences were generated using an algorithm called MultAlin of both varieties of cucumber plant, Chinese long and Krak. The color red indicates essentially the same in both sequences, with a few differences in some individual nucleotides marked in blue and very small fragment, which occurs only in the sequence generated, distinguished in black.

4.2.2 MTP9 promoter

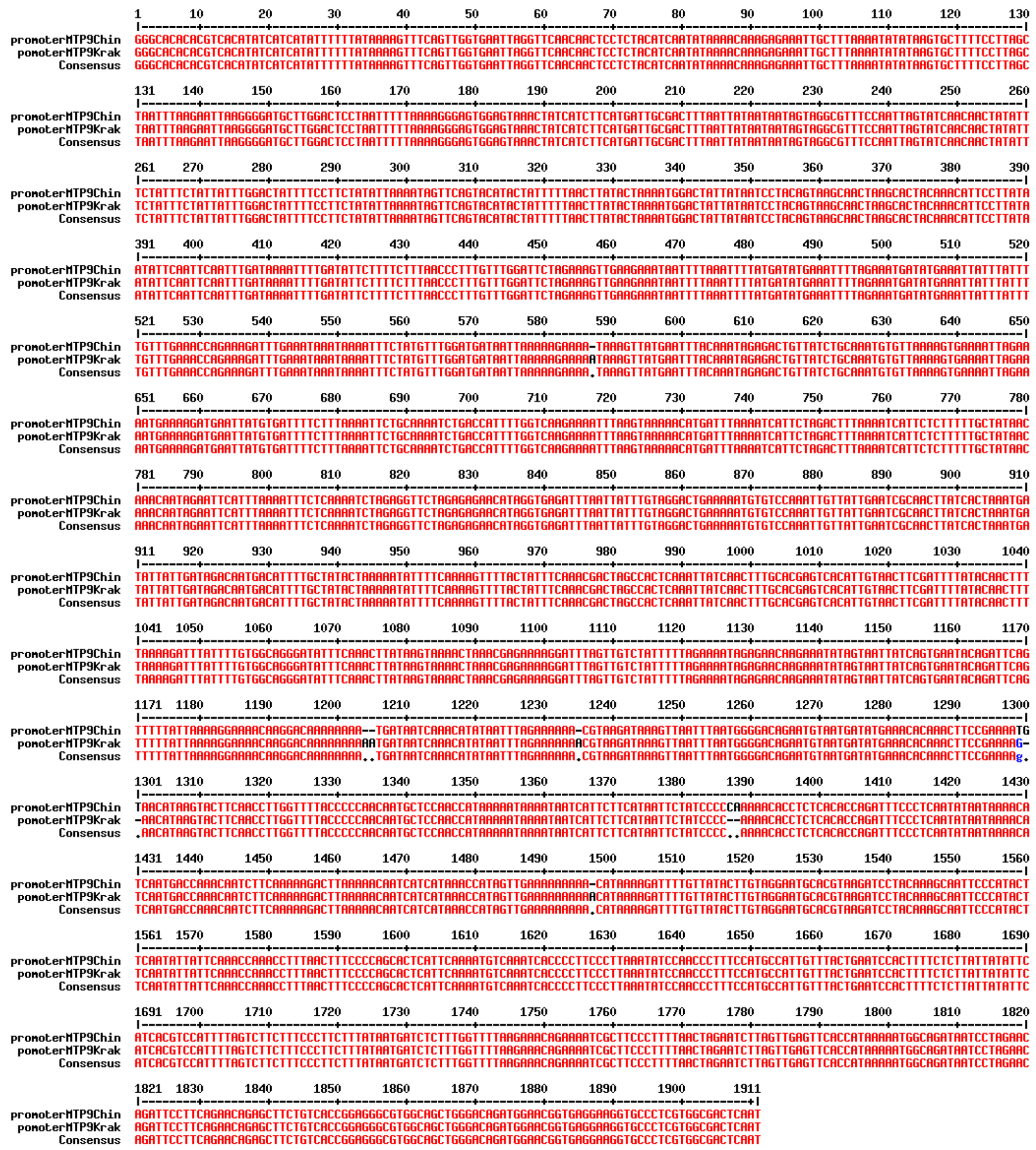

Figure 10. Comparison of the generated and obtained MTP9 promoter sequence. The predicted gene sequences were generated using an algorithm called MultAlin of both varieties of cucumber plant, Chinese long and Krak. The color red indicates essentially the same in both sequences, with a few differences in some individual nucleotides marked in blue and very small fragment, which occurs only in the sequence generated, distinguished in black.

4.3 Identification of the regulatory cis elements within the promoter sequences of cucumber genes *CsMTP6* and *CsMTP9*

The primary step for the functional analysis of the promoter is a database. In this case, we use plantCARE. The results given in MTP6 promoter are shown in the following tables for MTP6 and MTP9:

4.3.1 MTP6 promoter

Factor or site name	Frequency	Sequences	Function
5UTR Py-rich stretch	2	TTTCTTCTCT	conferring high transcription levels
AAGAA-motif	2	GAAAGAA	
ACE	1	AAAACGTTTA	cis-acting element involved in light responsiveness
ARE	1	TGGTTT	cis-acting regulatory element essential for the anaerobic induction
AT-rich element	1	ATAGAAATCAA	binding site of AT-rich DNA binding protein (ATBP-1)
AT1-motif	1	ATTAATTTTACA	part of a light responsive module
BOX 4	2	ATTAAT	part of a conserved DNA module involved in light responsiveness
BOX 1	8	TTTCAAA	light responsive element
BOX W1	2	TTGACC	fungal elicitor responsive element
CAAT-box	49	CAAT CAATT CAAAT CCAAT	common cis-acting element in promoter and enhancer regions
ERE	3	ATTTCAAA	ethylene-responsive element

G- BOX	1	CACGAC	cis-acting regulatory element involved in light responsivenes
GA-motif	2	AAAGATGA	part of a light responsive element
GAG-motif	2	AGAGATG	part of a light responsive element
GARE-motif	3	AAACAGA TCTGTTG	gibberellin-responsive element
HSE	2	AAAAAATTTC	cis-acting element involved in heat stress responsiveness
Skn-1_motif	1	GTCAT	cis-acting regulatory element required for endosperm expression
I-BOX	2	GATATGG	part of a light responsive element
LAMP-element	1	CCAAAACCA	part of a light responsive element
Sp1	4	CC(G/A)CCC	Light responsive element
TA-rich region	4	TATATATATATATATATATATA	enhancer
TATA-box	131	TTTTA TTTAAAAA TAATA ccTATAAAaa TATAAAA TATAAA TATAA ATATAT TATATTTATATTT TATAAATA TATAAAT TATAAATT TATAAATATAAA TATATAA TATATATA	core promoter element around -30 of transcription start

TATCCAT/C-motif	2	TATCCAT	
TC-rich repeats	1	ATTCTCTAAC	cis-acting element involved in defense and stress responsiveness
TCA-element	2	CAGAAAAGGA CCATCTTTTT	cis-acting element involved in salicylic acid responsiveness
TCT-motif	1	TCTTAC	part of a light responsive element
Unnamed__1	1	CGTGG	
Unnamed__3	1	CGTGG	
Unnamed__4	8	CTCC	
Unnamed__6	3	taTAAATATct	
WUN-motif	1	TCATTACGAA	wound-responsive element
as-2-box	1	GATAatGATG	involved in shoot-specific expression and light responsiveness
W box	2	TTGACC	
circadian	5	CAANNNNATC	cis-acting regulatory element involved in circadian control

4.3.2. MTP9 promoter

Factor or site name	**Frequency**	**Sequences**	**Function**
AAGAA-motif	3	gGTAAAGAAA GAAAGAA	
ABRE	2	CGTACGTGCA TACGTG	cis-acting element involved in the abscisic acid responsivenes

ARE	5	TGGTTT	anaerobic induction
BOX 1	5	TTTCAAA	light responsive element
BOX W1	1	TTGACC	fungal elicitor responsive element
CAAT-box	32	CAAT CAATT CAAAT CCAAT	common cis-acting element in promoter and enhancer regions
CAT-box	1	GCCACT	cis-acting regulatory element related to meristem expression
CATT-motif	1	GCATTC	part of a light responsive element
CGTCA-motif	1	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
ERE	3	ATTTCAAA	ethylene-responsive element
G- BOX	4	CACGTC CACGTA TACGTG	cis-acting regulatory element involved in light responsivenes
GARE-motif	1	AAACAGA	gibberellin-responsive element
GA-motif	1	AAAGATGA	part of a light responsive element
GT1-motif	1	GGTTAA	light responsive element
HSE	2	AAAAAATTTC	cis-acting element involved in heat stress responsiveness
I-BOX	3	ATGATATGA	part of a light responsive element
LTR	1	CCGAAA	cis-acting element involved in low-temperature responsiveness
MBS	1	CAACTG	MYB binding site involved in drought-inducibility

MRE	1	AACCTAA	MYB binding site involved in light responsiveness
Skn-1_motif	2	GTCAT	cis-acting regulatory element required for endosperm expression
O2-site	1	GATGATATGG	cis-acting regulatory element involved in zein metabolism regulation
TATA-box	97	TTTTA TTTAAAAA TAATA ccTATAAAaa TATAAAA TATAAA TATAA ATATAT TATATTTATATTT TATAAATA TATAAAT TATAAATT TATAAATATAAA TATATAA TATATATA	core promoter element around -30 of transcription start
TC-rich repeats	2	ATTTTCTTCA	cis-acting element involved in defense and stress responsiveness
TCA-element	2	GAGAAGAATA CAGAAAAGGA	cis-acting element involved in salicylic acid responsiveness
TCT-motif	2	TCTTAC	part of a light responsive element

TGA-element	1	AACGAC	auxin-responsive element
TGACG-motif	1	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness
Unnamed__13	1	TCCAAGTATA	
Unnamed__2	1	AACCTAACCT	
Unnamed__4	5	CTCC	
W box	1	TTGACC	
chs-CMA1a	1	TTACTTAA	part of a light responsive element
chs-CMA2a	1	GCAATTCC	part of a light responsive element
Circadian	3	CAANNNNATC CAAAGATATC	cis-acting regulatory element involved in circadian control

5. Discussion

As we can demonstrate, there are a few contrasts between promoter sequences from varieties Krak and Chinese Long cucumber plants. This may demonstrate for adjustment to the temperature European atmosphere conditions (lower normal light force and high temperatures). It is critical to realize that Chinese Long cultivar has more qualities identified with photooxidative anxiety resilience (UV and high light illumination), higher non-ideal temperatures resistance (e.g. heat shock proteins) and pathogen assault resistance (e.g. peroxidases and glycoproteins), in this way it is better adjusted to the subtropical atmosphere conditions. European cucumber cultivar has a much bigger number of qualities which join in ammonium particle osmosis. This is relied upon to adjust for the constrained capacity to absorb nitrate particles, most likely as an aftereffect of more elevated amounts of nonstop CO_2 emission in Europe till the 90's of the twentieth century. It appears like the time required by multicellular cucumber plants to adjust to new natural conditions is too short and constitute the new proof of "development in real life".

There are numerous *cis*-regulatory elements needed for the regulation of gene expression, which are areas of non-coding DNA which regulate their interpretation working as binding sites for transcription factors. As such, they are DNA acknowledgment successions which

connect with fundamental transcription initiation complexes and various transcription elements.
The control regions of cucumber plants promoter incorporate different cis-acting components that help to the complex expression profile of *CsMTP6* and *CsMTP9* genes. Besides, the same transcription factors can go about as activators or repressors relying upon their focus and the vicinity of collaborating partner proteins. While a large portion of these regulatory components are all around characterized today, there is minimal rationale clear in the association of various regulatory components, and even less in the way that they communicate to regulate gene expression.

In MTP6 and MTP9 promoters contemplated, all the needed data to coordinate legitimate gene expression is for the most part situated in an extremely minimal area. Just few plant promoters are known not constitutively communicated in most plant cells. Association of the upstream regulatory components with sequence-specific transcription factor decides the time, place and level of movement of all genes inside of an arrangement of profoundly facilitated expression systems.

There were found many *cis*-regulatory elements, which are described in the tables above, MTP6 both as MTP9. The most importants function of them are described below:

- **Light-responsive elements (LREs)**

Plants have built up a complex biochemical framework to see and react to light, which is in light of three classes of photoreceptors: phytochromes, blue-light receptors and UV-light receptors. Light flags consumed by these photoreceptors manage transcription of genes that encode proteins included in photosynthesis and different formative procedures through an unpredictable sign transduction course. There were discovered motifs, for example, the GT1-box (GGTTAA), I-box (GATAAAGR), G-box (CACGTG) and H-box, ACCTA(A/C)C(A/C), which have been tentatively demonstrated to be imperative segments in the light reaction. It has additionally been demonstrated that just fake sequences made out of matched combinations of tetrameric rehashes of G-and GATA-boxes, or GT1-and GATA-boxes, but not multimers of a solitary theme, can work as LREs. The measured segment of LREs is made out of two general components, the light-particular component, and a coupling component. Light-particular components are focused by transcription factors that are managed by light. Coupling components bind protein factors that direct the light boost to

transcription in a spatial or worldly way, or focus the relative quality of the light-incited gene expression.

- About *metabolic regulation,* it was demonstrated that hormones assume a key part in regulation cucumber development and improvement. Additionally, late confirmation has proposed that sugars control gene expression and formative procedures in a way like traditional plant hormones. There are a few hormones and responsive components that showed up in our study like vital components who begin cucumber promoters MTP6 and MTP9 action. The way they work can be summarized in:

- **Auxin-Responsive Elements (AuxREs)**

Auxin assumes an essencial part in root development, apical predominance, tropism, and senescence at the living being level, while acting as a signal for division, augmentation, and separation inside the plant cell. Inside of the composite AuxREs, auxin-responsive components repress the transcription-stimulating activity of the adjoining or covering constitutive component when auxin level is low. At the point when auxin level is high, the repression is discharged and the composite component is initiated. Depending the way of the constitutive or coupling components of diverse AuxREs, they could conceivably give a mixture of tissue-particular and formatively controlled expression

- **Gibberellin-Responsive elements (GAREs)**

The system by which the gibberellic acid (GA) sign is seen and transduced in plants has been considered widely. Here there are incorporated GARE motif (TAACA(A/G)A) and TATCCAC-box.

- **Abcisic Acid-Responsive elements (ABREs)**

Various ABREs are found upstream of the ABA-instigated genes. The G-box with the succession (C/G/T)ACGTGGGG is presumably the most known component subject to ABA regulation. Likewise with auxins, organ-and species-particular actuation of ABA-responsive genes is accomplished just by the helpful activity of a few cis-acting elements.

- **Ethylene-Responsive Elements (EREs)**

The best-known impact of ethylene is the advancement of natural product maturing. Other outstanding procedures managed by ethylene incorporate seed germination, senescence and reactions to stress factor, for example, flooding, injuring or pathogen assault.

- About *abiotic stress*, it was found:

- **Heat-Stress Responsive Elements (HSEs)**

The heat-stress reaction is generally rationed in every single living cell and heat shock transcription components are the focal proteins in this procedure. Notwithstanding their general variability in arrangement and size, their structure and promoter acknowledgment sequences are amazingly saved among the eukaryotes.

Every one of them embody a N-terminal DNA-tying area, the hydrophobic center of which ensures the exact area of the focal helix-turn-helix motif at the HSE. This component contains a dull example of palindromic binding motifs, nGAAnnTTCnnGAA and assumes a noteworthy part in the heat-stress reaction.

- **Oxidative-Stress Responsive Elements**

Expanding studies shows that H_2O_2 capacities as an anxiety motion in plants interceding versatile reactions to different stresses by tweaking articulation of numerous genes. A few promoter components, for example, W-, G-and H-boxes, and the ethylene-responsive GCC component, which are available in the oxidative anxiety responsive piece of plant promoters, have been set forward as possibility for H_2O_2-capable motifs.

- **Cold-, Drought- and Osmotic Stress-Responsive Elements**

At the point when presented to low temperatures, plant cells experience three big issues: changes in the spatial association of biological membranes, hindrance of biochemical and concoction responses, and adjustment in the accessibility and status of water. Accordingly a significant number of the adjustments in the gene expression that happen because of low temperature and dry season oblige ABA signaling, free signal transduction pathway. Drought and cold stress inducible genes that are enacted in this pathway containing a potential ABRE (T/C)ACGTGGG in MTP6 promoter locales.

Complex sub-atomic reactions to different drying out and cold-related stresses may be interceded by both regulatory systems, where the ABA-signalling pathway doesn't incite a quickly reaction, but rather assumes an essential part in delayed, long-term versatile reaction to cold stress instigated by dehydration of plant tissues.

- **Anaerobic-Responsive Elements (AREs)**

The low-oxygen reaction of higher plants is mind boggling and includes actuation of particular genes sets.

About *biotic stress* and *wounding*, it was found that pathogen and wound-incited signal transduction pathways in plants unite in the cell core. There is an extensive number of known pathogen-inducible qualities. Upstream regulatory sequences settled in these promoters shape a complex combinatorial regulation system and react to a several factors, including ethylene, salicylic acid (SA), ABA and different bacterial and contagious fungi.

6. Conclusion

This project is aimed at showing the qualities and intricacies of cucumber plant regulatory sequences and high lighting how their connections administer the structural and functional interplay of signal observation pathways. In the course of the most recent couple of years, an impressive collection of proof has gathered on the basic association and regulation of plant promoters, numerous parts of which have been already evaluated.

In this work, we have attempted to summarize both the well-known and recently depicted *cis*-active regulatory elements in the cucumber MTP6 and MTP9 promoters, intentionally accentuating the latest studies that light up their functional movement in the multi-level regulation of gene expression. Functional investigations of promoters have additionally offered ascent to a general idea that upstream regulatory elements are composite and not individual, where each *cis*-acting component helps to the general action of the module through synergistic associations between transcription factors. Particular components inside of a module are focused by transcription factors that are controlled by sign recognition pathways, while coupling components bind protein calculates that focus expression.

The practial significance of better comprehension the regulation of plant promoters is the possibility to impact gene expression to control plant digestion system and accomplish compartmentalized generation of valuable from roots, hypocotyl, cotyledons, leaves and petioles.

7. References

- Barbara Montanini, Damien Blaudez, Sylvain Jeandroz, Dale Sanders and Michel Chalot*[1] **Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity.**
- Jeffery L Gustin, Michael J Zanis and David E Salt **Structure and evolution of the plant cation diffusion facilitator family of ion transporters.**
- Felipe K. Ricachenevsky, Paloma K. Menguer, Raul A. Sperotto, Lorraine E. Williams and Janette P. Fett **Roles of plant tolerance proteins (MTP) in metal storage and potential use in biofortification strategies.**
- Hall JL, Williams LE: **Transition metal transporters in plants.** *J Exp Bot* 2003, **54(393):**2601-2613.
- Kambe T, Yamaguchi-Iwai Y, Sasaki R, Nagao M: **Overview of mam- malian zinc transporters.** *Cell Mol Life Sci* 2004, **61(1):**49-68.
- Gaither LA, Eide DJ: **Eukaryotic zinc transporters and their reg- ulation.** *Biometals* 2001, **14(3-4):**251-270.
- Nies DH: **Efflux-mediated heavy metal resistance in prokary- otes.** *FEMS Microbiol Rev* 2003, **27(2-3):**313-339.
- Nies DH, Silver S: **Ion efflux systems involved in bacterial metal resistances.** *J Ind Microbiol* 1995, **14(2):**186-199.
- Chao Y, Fu D: **Kinetic study of the antiport mechanism of an Escherichia coli zinc transporter, ZitB.** *J Biol Chem* 2004, **279(13):**12043-12050.
- Grass G, Otto M, Fricke B, Haney CJ, Rensing C, Nies DH, Munkelt D: **FieF (YiiP) from Escherichia coli mediates decreased cel- lular accumulation of iron and relieves iron stress.** *Arch Micro- biol* 2005, **183(1):**9-18.
- Kim D, Gustin JL, Lahner B, Persans MW, Baek D, Yun DJ, Salt DE: **The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator Thlaspi goesingense acts to enhance efflux of Zn at the plasma membrane when**

expressed in Saccharomyces cerevisiae. Plant J 2004, 39:237-251.

- Kobae Y, Uemura T, Sato MH, Ohnishi M, Mimura T, Nakagawa T, Maeshima M: Zinc transporter of Arabidopsis thaliana AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. Plant Cell Physiol 2004, 45:1749-1758.
- Muthukumar B, Yakubov B, Salt DE: **Transcriptional activation and localization of expression of Brassica juncea putative metal transport protein BjMTP1**. BMC Plant Biology 2007, 7:32.
- Grennan AK: **Genevestigator. Facilitating Web-Based Gene-Expression Analysis.** Plant Physiol 2006, 141:1164-1166.
- Lyons E, Freeling M: **How to usefully compare homologous plant genes and chromosomes as DNA sequences.** Plant J 2008, 53:661-673.
- Delhaize E, Kataoka T, Hebb DM, White RG, Ryan PR: Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. Plant Cell 2003, 15:1131-1142.
- Delhaize E, Gruber B, Pittman J, White R, Leung H, Miao Y, Jiang L, Ryan P, Richardson A: **A role for the AtMTP11 gene of Arabidopsis in manganese transport and tolerance**. Plant J 2007, 51:198-210.
- Peiter E, Montanini B, Gobert A, Pedas P, Husted S, Maathuis FJM, Blaudez D, Chalot M, Sanders D: **A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance**. Proc Natl Acad Sci USA 2007, 104:8532-8537.
- http://cucumber.genomics.org.cn/page/cucumber/index.jsp
- http://indousagriseeds.com/index.php?option=com_content&view=article&id=116&Itemid=141
- Slavko Komarnytsky and Nikolai Borisjuk, **Functional analysis of promoter elements in plants**
- Clemens S, Bloss T, Vess C, Neumann D, Nies DH, Zur Nieden U: **A transporter in the endoplasmic reticulum of Schizosaccha- romyces pombe cells mediates zinc storage and differen- tially affects transition metal tolerance.** *J Biol Chem* 2002, **277(20):**18215-18221.
- Grunberg K, Wawer C, Tebo BM, Schuler D: **A large gene cluster encoding several magnetosome proteins is conserved in dif- ferent species of magnetotactic bacteria.** *Appl Environ Microbiol* 2001, **67(10):**4573-4582.
- Li L, Kaplan J: **Characterization of two homologous yeast genes that encode**

mitochondrial iron transporters. *J Biol Chem* 1997, **272(45):**28485-28493.

- Peiter E, Montanini B, Gobert A, Pedas P, Husted S, Maathuis FJM, Blaudez D, Chalot M, Sanders D: **A secretory pathway-localized cation diffusion facilitator confers plant manganese toler- ance.** *Proc Natl Acad Sci U S A* in press.
- Desbrosses-Fonrouge AG, Voigt K, Schroder A, Arrivault S, Thomine S, Kramer U: **Arabidopsis thaliana MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation.** *FEBS Lett* 2005, **579(19):**4165-4174.
- Kim D, Gustin JL, Lahner B, Persans MW, Baek D, Yun DJ, Salt DE: **The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator Thlaspi goesingense acts to enhance efflux of Zn at the plasma membrane when expressed in Sac- charomyces cerevisiae.** *Plant J* 2004, **39(2):**237-251.
- Pittman JK: **Managing the manganese: molecular mechanisms of manganese transport and homeostasis.** *New Phytol* 2005, **167(3):**733-742.
- Gonnet GH, Cohen MA, Benner SA: **Exhaustive matching of the entire protein sequence database.** *Science* 1992, **256(5062):**1443-1445.
- Magali Lescot, Patrice D�hais, Gert Thijs, Kathleen Marchal, Yves Moreau, Yves Van de Peer, Pierre Rouz� and Stephane Rombauts *Nucleic Acids Res. 2002 Jan 1;30(1):325-327.* **PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences.**
- F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890. **MultAlin "Multiple sequence alignment with hierarchical clustering" .**