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Genetics / Genética

In silico identification and expression analysis of Metal-nicotianamine transporter (YSL_3) and Oligopeptide transporter 3 (OPT_3) under Cd stress in *Brassica oleracea var. acephala*

Identificación *in silico* y análisis de expresión del transportador de metal-nicotianamina (YSL_3) y el transportador de oligopéptidos 3 (OPT_3) bajo estrés de Cd en *Brassica oleracea* var. *acephala*

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Abstract

Background: Metal-nicotianamine transporter (YSL) family protein belongs to the oligopeptide heavy metal transporter group, as characterized in *Arabidopsis thaliana*. Oligopeptide transporters (OPTs) are a group of membrane-localized proteins, involved in different transport mechanisms, contributing to nitrogen mobilization, glutathione transport and long-distance metal distribution. Metal-nicotianamine transporter gene 3 (YSL_3) incorporates the oligopeptide transporter domain, found to transfer several heavy metals in diverse plant species, and among them cadmium transport in *Brassica oleracea*.

Objective: To evaluate and confirm the expression of Metal-nicotianamine transporter (YSL_3) under cadmium stress.

Studied species: *Brassica oleracea* var. *acephala*

Study site and dates: *Brassica oleracea* var. *acephala* samples were collected from Blagaj region, Bosnia and Herzegovina.

Methods: Through a simple bioinformatic approach the interactome partner of Metal-nicotianamine transporter (YSL_3) was discovered and annotated. Oligopeptide transporter 3 (OPT_3) and Metal-nicotianamine transporter (YSL_3) genes were checked for expression levels under cadmium stress.

Results: We have identified a strong interacting partner of YSL_3, later confirmed as Oligopeptide transporter 3 (OPT_3) protein in *Brassica oleracea*. The in vitro expression analysis by using a qRT-PCR revealed a significant upregulation of YSL_3 and OPT_3, during Cd stress.

Conclusions: These findings indicate that the represented *in-silico* approach, followed by *in vitro* gene expression study, successfully confirmed YSL_3 and identified OPT_3 as a new gene, in correlation to cadmium stress.

Keywords: Bioinformatics, Gene expression, Heavy metals, Uncharacterized protein.

Resumen

Antecedentes: La proteína transportadora de metales quelados con nicotianamina (yellow-stripe-like -YSL) pertenece a la familia de transportadores de oligopéptidos, caracterizada en *Arabidopsis thaliana*. Los oligopéptidos transportadores (TOPS) son un grupo de proteínas localizadas en la membrana, involucrados en movilizar nitrógeno, transportar glutatión y distribuir metales a larga distancia. El gen para el transportador de metal-nicotianamina 3 (YSL_3) incorpora un dominio transportador que ha sido reportado en la transferencia de varios metales en diversas especies, entre ellos el transporte de cadmio en *B. oleracea.*

Objetivo: Evaluar y confirmar la expresión del transportador de metal-nicotianamina (YSL_3) bajo estrés de cadmio.

Especie estudiada: *Brassica oleracea* var. *acephala*

Sitio de estudio y fecha: Las muestras de *B. oleracea* var. *acephala* se recolectaron en Blagaj region, Bosnia y Herzegovina.

Métodos: Mediante un enfoque bioinformático simple se descubrió y anotó el interactoma del transportador de metal-nicotianamina (YSL_3) y se determinaron los niveles de expresión de TOP_3 y YSL_3 por medio de qRT-PCR.

Resultados: Hemos identificado un compañero de interacción fuerte de YSL_3, más tarde confirmado como proteína transportadora de oligopéptido 3 (TOP_3) en *B. oleracea*. El análisis de expresión in vitro mediante el uso de una qRT-PCR reveló una regulación positiva significativa de YSL_3 y TOP_3, inducida por el estrés de Cd.

Conclusiones: Estos hallazgos indican que el enfoque representado *in-silico*, seguido del estudio de expresión génica *in vitro*, confirmó con éxito YSL_3 e identificó a TOP_3 como un nuevo gen de *Brassica oleracea*, en correlación con el estrés de cadmio.

Palabras clave: bioinformática, proteína no caracterizada, expresión génica, metales pesados.

Brassica oleracea is a member of the Brassicaceae family, which according to the recent findings has 341 genera and 3,977 species (Franzke *et al.* 2011). Kale (*Brassica oleracea* var. *acephala*) is one of the oldest forms of the *Brassica oleracea* varieties that originate from the eastern Mediterranean (Balkaya & Yanmaz 2005). It is a diploid species with CC genome type and 9 haploid chromosomes (Sun *et al.* 2019). It has an important place in the diet of the people worldwide and it has been used in traditional medicine due to its anticancerogenic and antioxidant properties (Parkin *et al.* 2014). *Brassica* species, including *Brassica oleracea* var. *acephala*, are considered as potential phytoextraction plants since they are known to accumulate high amounts of toxic heavy metals in their tissues, without visible symptoms (Gall *et al.* 2015).

Cadmium is one of the common toxic heavy metals that exist at low concentrations in soil under natural conditions. It is included in the production of fertilizers, alloys, detergents, batteries, petroleum, and pigments (Raymond & Okieimen 2011). Its toxicity potential effects soil, water, air, and plants, therefore, contaminating the food chains. Cadmium uptake disturbs photosynthesis, enzyme activities, decreases nutrient, and water uptake, inhibiting the growth rate of the plants (Fahad *et al.* 2017).

The key mechanism in the protection and survival of plants under heavy metal stress is regulation of its gene expression (Dutta *et al.* 2018b). YSL3 belongs to Oligopeptide transporter family (OPT), known to be involved in different processes nitrogen mobilization (Koh *et al.* 2002, Cagnac *et al.* 2004, Stacey *et al.* 2008), glutathione transport (Cagnac et al. 2004, Zhang *et al.* 2004), heavy metal sequestration (Vasconcelos et al. 2008), and long-distance metal distribution (Stacey et al. 2008).

According to Feng *et al.* (2017), Metal-nicotianamine transporter 3 (YSL_3) gene expression increased significantly in response to excess cadmium in *Solanum nigrum*. On the other hand, the response of genes to metal-deficient, metal-sufficient or metal-excess conditions is partially depending on the plant species (Morkunas *et al.* 2018). In this regard, the Cd stress-induced expression of YSL_3 in correlation to an *in-silico* predicted oligopeptide transporter 3 (OPT_3), was analyzed. The YLS_3 and OP_{T3} expression levels might be an effective approach for understanding cadmium accumulation in *Brassica oleracea* and enhancing heavy metal tolerance.

Material and methods

Sequence prediction and *in-silico* interactome identification of OPT3. The whole genomic sequence of *Brassica olaracea* (B.O) was published in 2013, currently available at the Brassicae database (Cheng *et al.* 2011). This database incorporates an option for Basic Local Alignment Search Tool (BLAST) from the *Arabidopsis thaliana* genome. Basically, all genes used in this study were annotated from *A. thaliana* genome, extracted from NCBI genome browser, and blasted as FASTA sequences to Brassica database (Cheng *et al.* 2011), to find the homologues genes in *B. olaracea* genome, later used for primer design.

STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database was used for Interactome analysis (Franceschini et al. 2012). The predicted YSL_3 (NCBI accession number: 106319276) was used as a query for the Interactome analysis. The significance and reliability of the search was kept at high level by the confidence score of 0.7. For additional and detailed genomic information of predicted proteins, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was accessed (Kanehisa & Goto 2000).

Primer design for *Brassica olaracea* genes. The predicted *B. olaracea* gene sequences, later annotated as YSL3 and OPT, were used as template sequences for the prediction of corresponding primers, required for Real-Time PCR analysis. Based on a study from 2014, the housekeeping gene Ubiquitine2 emerged as one of four genes, being very stable across the tested conditions for gene expression analysis (Brulle *et al.* 2014) (see Table 1). For the primer design, an incorporated tool within the NCBI server, called PRIMER-BLAST was used (Ye *et al.* 2012). The selected primers were chosen upon the following parameters: min annealing temperature of 57 ºC, maximum of 62 ºC and optimum of 60 ºC, 50 to 60 % of GC, and the primers should be 18-22 bp in length. In Figure 1 the schematic presentation of all bioinformatics protocols used in this study is shown.

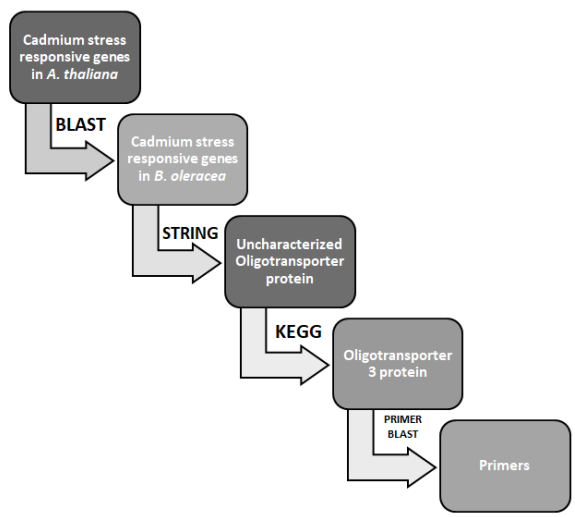

Figure 1. Schematics of the bioinformatic protocol used in this study

Table 1. Oligonucleotides used in in the present research

Genes	Sequences (5'->3')
Oligopeptide transporter gene 3 (OPT3)	F AGCTTCCATGGTGGGGAATG
	R ACCAGCCAGCTAGCAATGTT
Metal-nicotianamine transporter 3 (YSL3)	F TTCCTGGGGAATCATGTGGC
	R CCCGTCTCCGAGGATCAATG
Ubiquitin2 (UBQ2)	F ATATTCGTGAAGACGCTG
	R CTCAACTGGTTGCTGTG

Plant growth and root assay analysis under cadmium treatment. In the study, a domestic kale variety, marked as kale 1, was obtained from Blagaj region, near Mostar city - Bosnia and Herzegovina (43° 25 North,17° 88 East). *Brassica oleracea* var. *acephala* seedlings were grown using Tap of paper method. Seeds were treated with different concentrations of cadmium (50, 100, 200, and 500 µM) and incubated for 5 days at 27 ºC in growth chamber where they were exposed to light for 16 hours a day. When plants reached their sufficient growth they were removed carefully from the paper and root length was measured with a ruler. After the root length measurement, the numbers, indicated in centimeters, were entered for statistical analysis. All samples were stored at -80 ºC prior RNA isolation.

RNA isolation and RT-qPCR analysis. The total RNA isolation was performed based on a standard CTAB protocol (Márquez 2005), but slightly optimized. New optimized procedure was based on homogenization tissue in CTAB extraction buffer and selective precipitation of RNA with LiCl (see Appendix 1). RNA concentration and purity was determined using Thermo Scientific µDrop Plate and RNA integrity was confirmed by 1 % agarose gel. Isolated RNA was stored at -80 ºC. The presence of contaminating genomic DNA can give false positives in qPCR, therefore the isolated RNA was treated with DNAse prior to cDNA synthesis. DNAse I (RNAse-free) kit from Thermo Fisher Scientific was used according to manufacturer's instructions. Complementary DNA (cDNA) is generated from an RNA template by reverse transcription using SCRIPT cDNA Synthesis Kit from Jena Bioscience according to manufacturer's instructions. qPCR amplification was performed on the StepOnePlus system by Applied Biosystems®. The reaction was prepared using the Maxima SYBR Green/ROX qPCR Master Mix by Thermo Scientific™ according to manufacturer's instructions. qPCR Thermal cycle was adjusted under the following conditions: PCR initial activation step 95 ºC for 0 min, followed by 35 cycles of 95 ºC for 15 s, 60 ºC for 30 s, and 72 ºC for 30 s.

Statistical analysis. Root length data were presented graphically as mean + standard deviation. Descriptive statistical analyses of root length results were performed using GraphPad Prism 18.3.0. Significant difference between each treatment was accepted if $p < 0.05$. Relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

Results

In-silico identification of genes involved in Cd stress. The *in-silico* interactome analysis showed that YSL_3, as a known Cd transporter, correlates with OPT and an unknown protein partner for which the annotation is not still available (blue node). (Figure 2). Further, through the additional analysis of uncharacterized OPT protein, with its accession number 106311671, in KEGG online database, we correlated this accession number to oligopeptide transporter 3 (OPT_3) protein. Therefore, its expression under cadmium treatment was further examined, for the first time considering OPT3 as a potential gene correlated to cadmium homeostasis in the *B. oleracea* plant.

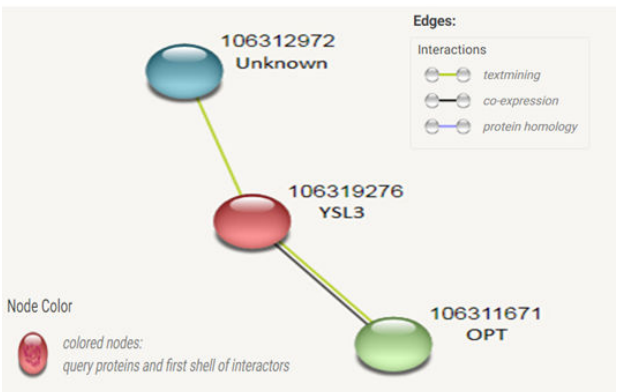

Figure 2. YSL_3 interactome analysis by STRING (0.7 confidence level)

Root essay analysis under cadmium treatment. The average root length of kale 1 can be observed in Figure 2. The root length decreased with the increase of $CdCl_2$ concentrations. In the control group, root length ranged from 2.8 cm to 3.8 cm, while in 500 µM Cd it ranged from 0.6 to 1.7 cm, with an average root length of 1.056 cm. Tukeys multiple comparisons test showed that there was a significant difference in the mean root length between the all concentrations used in the study, $p < 0.0001$ ($p < 0.05$) (Table 2). In order to summarize and present the whole data features, obtained by the root assay analysis, a descriptive statistics approach is shown in Supplementary material S. Table 1. An example of visual changes in root size is shown with kale 1 (see Supplementary material S. Figure 1).

Table 2. Tukeys multiple comparisons test for root lenght

Samples	Mean Diff.	Significant?	*p*-value
control vs. 50 µM	0.2879	Yes	0.0009
control vs. 100 µM	0.9061	Yes	0.0009
control vs. 200 µM	1.179	Yes	< 0.0001
control vs. 500 µM	2.148	Yes	< 0. 0001

$p < 0{,}0001$ ($p < 0.05$)

Genes expression analysis OPT and YSL_3 genes. Real-time PCR was used to determine the relative expression of the OPT_3 and YSL_3 genes, with 5 replicates. In addition, only the results whose melt curve generated a single peak were used, which means that only one product (gene of interest) was produced in the reaction. Melt curve analysis was performed to validate the specificity of the reaction by checking for primer-dimers or nonspecific amplifications. Data are presented graphically where Y-axis in graphs represents $2^{\wedge-\Delta\Delta Ct}$ which basically analyzes the relative changes in gene expression.

The effect of Cd on YSL_3 and OPT_3 gene expression is shown in Figure 3. Different expression patterns were observed at different concentrations. There is a linear increase in gene expression levels by increasing Cd concentrations. Cadmium stress-induced approximately 4-fold changes of OPT gene expression, and a 2.5-fold change of YSL_3 gene expression when treated with 500 µM Cd. Cadmium significantly increased the YSL_3 gene expression linearly. Our results show that different cadmium concentrations trigger the expression of the YSL_3 and OPT_3 genes in *B. oleracea var. acephala.*

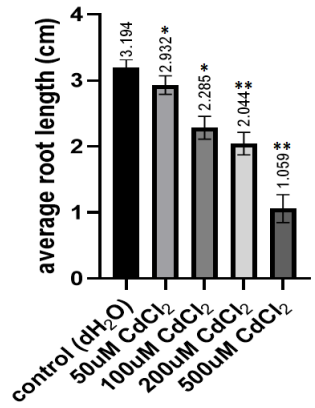

Figure 3. Average root length of kale 1. The average root length in *Brassica oleracea* under control-dH_20, 50, 100, 200, 500 µM of $CdCl_2$. Vertical lines represent the standard deviation of three biological replicates from root length measurements. Asterisks indicate the mean values that are significantly different between the treatment and control (*$p < 0.05$, **$p < 0.001$).

Discussion

Using an *in-silico* approach, the YSL_3 gene interactome analysis was performed, initially yielding a strong interaction to an uncharacterized OPT protein (106311671) (Figure 2). However, through a detailed KEGG pathway analysis, the uncharacterized OPT protein was annotated as Oligopeptide transporter 3 (OPT_3).

By analyzing the root lengths under different $CdCl_2$ concentrations, an initial effect of cadmium on plant root growth was observed. The results showed that the root length of kale 1 decreased by increasing cadmium concentrations as compared with control (Figure 3). With the increase of cadmium, further reduction in kales' root length was observed. These observations are in agreement with the results of Ahmad *et al.* (2015), where Cd affected most of the growth parameters in *Brassica juncea* including decreased shoot and root length. Nouairi *et al.* (2006) reported that the accumulation of Cd suppresses the growth of root and shoot in *B. juncea* and *B. napus* under cadmium stress. Further, Dutta *et al.* (2018b) reported also reduced root growth and shoot lengths in *B. juncea* under different heavy metals, including cadmium. Similar results were reported by Pandey & Ripathi (2011) where Cd treatment has an inhibitory effect on the growth of *Albizia proceraas* roots.

Brasscia oleracea is known due to its phytoremediation and phytoextraction capabilities (Mourato 2015), especially where high levels of Cd pollution exist. The genome of *B. oleracea* was fully published in 2016 (Lee *et al.* 2016), and its metabolism pathways regulating heavy metal stress still remain unknown. The *B. oleracea* variety used in this study was obtained from Blagaj region (Bosnia and Herzegovina), known to have high contaminated soil with heavy metals, especially the cadmium concentrations exceed limiting values (Sefo *et al.* 2010). Therefore, to specifically target the genes correlated to cadmium ions, the root assay protocol was optimized only to concentrate on cadmium ions. Growing the seed on paper towels with water and $CdCl_2$ solutions, we avoided any possibility to contaminate the seeds with other metals and conditions as pH, organic matter which is found in natural soil. It is known that the Cd intake is conditioned by its concentration in the soil, and availability of organic matter, pH, redox potential, temperature, and concentrations of other elements (Benavides *et al.* 2005). Roots are more affected in comparison to other organs since they are in direct contact with the toxic elements. The Cd triggers a set of complex changes in root morphology which later on through the apoplastic pathway disrupt the complete metabolism of the plant (Chen *et al.* 2011). This decline in total growth might be due to lowered water potential and nutrient imbalance caused by heavy metal stress (Jibril *et al.* 2017).

The results from qPCR gave insight into the relative gene expression of YSL_3 and OPT_3 genes in *B. oleracea* varieties under a specific level of Cd. In this research, the gene expression results suggest that Cd triggers the expression of the YSL_3 and OPT_3 genes. This is evident for all samples treated with $CdCl_2$ solution, as a significant decrease in root growth is seen with an increase in Cd concentrations (see Figure 3 and Table 2). The gene expression levels rise linearly with the increase of Cd concentrations, indicating that these genes might play a role in Cd homeostasis in *B. oleracea* (Figure 4). Wang *et al.* (2013) reported that over-expression of YSL in tobacco plants improves metal tolerance of the plant and, more specifically, increases the root-to-shoot translocation of Cd. YSL_3 expression increased significantly in response to excess cadmium in *S. nigrum, where* YSL_3 over-expression decreased cadmium accumulation in *B. oleracea* roots (Feng *et al.* 2017). The expression levels of OPT_3 gene changed similarly as YSL_3 gene. In *Arabidopsis thaliana*, the Oligopeptide Transporter 3 is known to be the main transporter of Fe into the phloem (Khan *et al.* 2018). Besides iron deficiency regulation, studies on OPT3-T-DNA mutants showed that OPT_3 might be involved in embryo *A. thaliana* development (Stacey *et al.* 2008). In regards to cadmium accumulation, until now only one study demonstrated that knocking out the OPT_3 gene in *A. thaliana* led to the over-accumulation of cadmium in seeds (Mendoza-Cózatl *et al.* 2014). In a recent study conducted in 2019 on peanut plants (*Arachis hypogaea* L.), the OPT_3 gene is verified to be responsible for Cd uptake and translocation (Chen *et al.* 2019). However, this study represents an initial attempt to correlated OPT_3 gene expression with cadmium accumulation in *B. oleracea species,* where a 4-fold overexpression of OPT_3 is noticed if compared to the control (Figure 4).

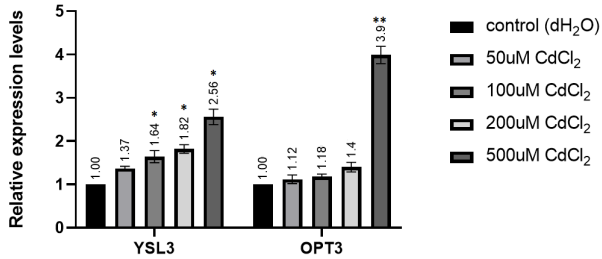

Figure 4. The effect of Cd on relative expression levels of YSL_3 and OPT_3. The relative gene expression levels in total biomass in *Brassica oleracea* under control-dH_20, 50, 100, 200, 500 µM of Cd. The gene was quantified by qPCR and normalized with the housekeeping gene Ubiquitin2 transcript. Vertical lines represent the standard deviation of three biological replicates from Ct values of independent experiments. Asterisks indicate the mean values that are significantly different between the treatment and control (*$p < 0.05$, **$p < 0.001$).

For the first time, an analysis of the expression of cadmium-related OPT_3 and YSL_3 genes in *B. Oleracea* were performed and correlated. As seen in Figure 1, through a simple *in-silico* approach YSL_3 was determined to be correlated with OPT_3. In essence, the expression level of both genes was in a linear expression pattern with increasing the Cd concentration. Different concentrations triggered a significant over-expression of the YSL_3 and OPT_3 genes in comparison to the control, with a 2.5 to 4-fold change at 500 µM $CdCl_2$ concentrations. However, further analysis should be undertaken to additionally confirm the correlation of cadmium YSL_3 and OPT_3 gene expression levels.

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Appendix 1

- Frozen samples of plant (0.5 g) were finely grounded using mortar and pestle together with liquid nitrogen.
- Transferred grinned tissues to 15 mL tube containing 2 ml of CTAB based extraction buffer warmed prior to 65 °C at water bath (see Supplementary material S. Table 2).
- 99 % β-Mercaptoethanol (30 µL) was added, and samples were placed at 65 °C for 30 min with gentle inversion every 5 minutes.
- An equal volume of Phenol: chloroform: isoamylalcohol (25:24:1) was added (in this case 2ml), mixed vigorously in a vortex for 30 s and incubated for 10 min at room temperature.
- The contents were centrifuged at 13,500 × g for 15 min at 4 °C.
- The aqueous supernatant was transferred to a new tube, and the Phenol: chloroform: isoamyl alcohol (25:24:1) extraction was repeated.
- To the upper phase, 4 M LiCl (final concentration, 2 M) was added, mixed well, and incubated at -80 °C for 1 h or overnight at -20 °C.
- RNA was pelleted at 12,000 × g and 4 °C for 20 min.
- The pellets were washed two times with 1 mL 80 % ethanol and air-dried.

The total RNA pellet was resuspended in 100 µL RNase-free water and stored at -80 °C until use.