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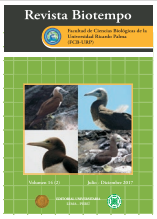

ORIGINAL ARTICLE / ARTÍCULO ORIGINAL

PLASMID DESING FOR THE EXPRESSION OF CYND IN *ARABIDOPSIS THALIANA* VIA *AGROBACTERIUM TUMEFACIENS*

DISEÑO DE PLÁSMIDOS PARA LA EXPRESIÓN DEL CYND EN *ARABIDOPSIS THALIANA* VÍA *AGROBACTERIUM TUMEFACIENS*

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ABSTRACT

Cyanide, used by the mining industries, is a toxic compound which mainly affects c-oxidase metalloenzyme in living beings, an essential enzyme for cellular respiration. The inhibition of this enzyme blocks oxidative phosphorylation, causing cell death. Biological methods such as phytoremediation provide an alternative to reduce or degrade contaminants such as cyanide. However, this technique would require plants that tolerate high concentrations of the metal present in the soil. On the other hand, some microorganisms have a high capacity of cyanide detoxification due to many metabolic pathways they have, such as *Bacillus pumilus* Meyer and Gottheil 1901, which presents the cyanid CynD that allows the degradation of cyanide formic acid and ammonium. The aim of this research was to design plasmids for the CynD expression in a model plant such as *Arabidopsis thaliana* (L.), Heynh. via *Agrobacterium tumefaciens* (Smith & Townsend, 1907) Conn, 1942 to lay the foundations to evaluate if the CynD of *B. pumilus* could confer to the plants the ability to grow in the presence of cyanide and assist in its degradation. For this purpose, bioinformatic tools were used to design a cloning vector (pUCCynD), inserting *in silico* the CynD sequence in the polylinker of the plasmid pUC18, among the EcoRi (5'GAATTC - 3'CTTAAG) and BamHi (5'GGATCC-3'CCTAGG) enzymes; and a transformation vector (pBCynD), inserting *in silico* the CynD sequence into the polylinker of the plasmid pBI121, among the ScaI (5'GAGCTC-3 'CTCGAG) and BamHI (5'GGATCC-3'CCTAGG) enzymes.

Keywords: *Agrobacterium tumefaciens* – Cyanide – CynD – genetic transformation

RESUMEN

El cianuro, usado por las industrias mineras, es un compuesto tóxico que afecta principalmente a la metaloenzima c oxidasa en los seres vivos, enzima esencial para la respiración celular; por lo que la inhibición de esta enzima bloquea la fosforilación oxidativa, provocando la muerte celular. Ante ello, métodos biológicos como la fitorremediación proporcionan una alternativa para reducir o degradar contaminantes como el cianuro; sin embargo, esta técnica requeriría plantas que toleren altas concentraciones del metal en el suelo. Por otro lado, algunos microorganismos tienen una alta capacidad de desintoxicación de cianuro debido a una serie de vías metabólicas, como es el caso del *Bacillus pumilus* Meyer and Gottheil 1901, que presenta la cianidasa CynD que permite la degradación del cianuro a ácido fórmico y

amonio. Por ello, el presente trabajo tiene como objetivo el diseño de plásmidos para la expresión de CynD en una planta modelo como la *Arabidopsis thaliana* (L.), Heynh. vía *Agrobacterium tumefaciens* (Smith & Townsend, 1907) Conn, 1942 con el fin de sentar las bases para evaluar si el CynD de *B. pumilus* le podría conferir a las plantas la facultad de crecer en presencia de cianuro y ayudar a su degradación. Para lo cual, se hizo uso de herramientas de la bioinformática, logrando diseñar un vector de clonación (pUCCynD), insertando *in silico* la secuencia del CynD en el polilinker del plásmido pUC18, entre las enzimas EcoRi (5'GAATTC - 3'CTTAAG) y BamHi (5'GGATCC-3'CCTAGG) del sitio múltiple de restricción y también un vector de transformación (pBCynD), insertando *in silico* la secuencia del CynD en el polilinker del plásmido pBI121, entre las enzimas ScaI (5'GAGCTC - 3'CTCGAG) y BamHI (5'GGATCC - 3'CCTAGG).

Palabras clave: *Agrobacterium tumefaciens* – Cianuro – CynD – transformación genética

INTRODUCTION

The growing interest in the exploitation of gold by various mining companies stems from increases in gold prices that provide a high profit margin and from the recent creation of cost-effective methods of production, such the extraction of gold in extremely poor deposits thanks to the technology of extraction by leaching with cyanide.

This technology has replaced the recovery of gold by amalgamation with mercury, since it allows a recovery of 97% of the mineral, compared to the 60% that mercury allows (Vargas, 2017). This ability of cyanide is due to its property of complexing with heavy metals.

The free cyanide comprises the hydrocyanic acid gas (HCN) and the cyanide ion (CN-) present in solution, but only the CN has the capacity to form complexes with different metal ions, for this reason this ion is used in industrial applications (Vargas, 2017; Donato *et al.*, 2007).

However, free cyanide (HCN and CN-) is extremely toxic. It mainly affects the metalloenzyme cytochrome c oxidase in living beings, an essential enzyme for cellular respiration. Therefore, the inhibition of this enzyme blocks oxidative phosphorylation, decreasing ATP concentration in the cell and causing cell death (Donato *et al.*, 2007). Cyanide may also inhibit the activity of at least 13 other enzymes, such as catalase, peroxidase, phosphatase, ribulose-1,5-bisphosphate, etc. (Vasil'ev *et al.,* 2007).

Considering that, biological methods such as phytoremediation provide an alternative to reduce or degrade contaminants such as cyanide. However, this technique would require plants that tolerate high concentrations of the metal in the soil.

Although plants have enzymes such as β-cyanoalanine synthase, to prevent their self-poisoning with cyanide they produce from hydrolysis of cyanogenic glycosides and as a by-product of ethylene biosynthesis, these enzymes only detoxify limited concentrations of exogenous cyanide (Logan *et al.*, 2000; Molojwane, 2015). This causes that on exposure to relatively low concentrations of exogenous cyanide, they die (Kebeish *et al.,* 2015; Molojwane, 2015). In contrast, some microorganisms have a high capacity for cyanide detoxification due to their many metabolic pathways (Gong *et al.,* 2012; Molojwane, 2015) such as *Bacillus pumilus* Meyer & Gottheil 1901, which presents the CynD cyanidase that allows the degradation of cyanide to formic acid and ammonium.

Therefore, if we want to apply techniques such as phytoremediation it is necessary to obtain plants that have resistance to high concentrations of cyanide and be able to use and degrade it (Molojwane, 2015). This is why we are working on the insertion of bacterial genes associated with the degradation of toxic tailings, such as cyanide, in a model plant: *A. thaliana*.

Genetic transformation of bacteria, is a laboratory procedure by which genetic material is introduced into a bacterium. Generally, the inserted genetic material is known as plasmid (circular DNA), but other forms of genetic material, such as DNA or RNA, may be inserted. The transformation has many applications, such as the production of proteins, the production of the same plasmids, the production of remedial bacteria, among others.

The plasmids used for the transformation of bacteria generally contain one or several genes of interest, a reporter gene and an antibiotic resistance gene, which enables the selection of the transformed bacteria from others by their ability to grow in medium containing the antibiotic resistance (Echenique, 2004).

The design of vectors for the genetic transformation of plants by the use of *Agrobacterium* species is widely used because only the T-DNA edge sequences are required for the transfer to take place (Garfinkel *et al.,* 1981; Zambryski *et al.,*1983). Some or all of the T-DNA bacterial genes can be removed by giving rise to unarmed vectors, which can transform plant cells without the general symptoms of bacterial infection.

During the infection process, *Agrobacterium tumefaciens* (Smith & Townsend, 1907) Conn, 1942 introduces into the plant cell a part of its DNA (transfer DNA) which is integrated into the genome of the plant (Binns & Campbell, 2001; Valderrama *et al.*, 2005; Rodríguez, 2012). T-DNA genes are expressed in their host and induce the formation of tumors and the synthesis of amino acid derivatives called opines which are exploited by the bacteria (Binns & Campbell, 2001). The T-DNA is located on the Ti plasmid (Tumor-inducing plasmid), which also contains the vir genes that are necessary for the transfer and incorporation of the DNA fragment into the genome of the plant (Binns & Campbell, 2001; Valderrama *et al.*, 2005; Rodríguez, 2012).

However, to make this real, the first step is to obtain a cloning vector that allows the replication of the gene of interest (CynD) and a transformation vector containing a genetic construct, allowing expression of the gene of interest in the plant (Gutarra, 2004; Jiménez, 2014; Vázquez, 2016). Traditionally these constructs have been developed using conventional molecular biology techniques, however at present we can make use of bioinformatic tools to perform the *in silico* design of the genetic construct and contract the synthesis thereof (Jiménez, 2014).

In the present project two bioinformatic tools like SnapGene and ApE were used, the first to download the plasmids that were used, visualize the codons, show in a linear and circular form the obtained plasmids and the second to visualize the ORFs, select the restriction enzymes and to design and visualize the primers.

The aim of this research was to design plasmids for CynD expression in *Arabidopsis thaliana* (L.), Heynh. mediated by *A. tumefaciens*.

MATERIALS AND METHODS

In this project we designed two types of plasmids that allow the transfer of the CynD gene to *Arabidopsis thaliana*, in order to lay the foundations to evaluate if the transformation of this plant with this gene coding for the enzyme cyanide dihydratase - which has the property of cyanide detoxification - can confer on plants the ability to grow in the presence of cyanide and aid in its degradation.

This requires the design of a plasmid that acts as a cloning vector to be able to replicate and obtain multiple copies of the CynD isolated from *B. pumilus*, and another plasmid that is used as a transformation vector to transfer the CynD from *A. tumefaciens* to *A. thaliana*.

Modification of the coding sequence

First, it was determined which genetic sequence was to be used for the construction of the plasmids. The cyanide dihydratase (CynD) gene sequence of *B. pumilus* strain C1 (1381pb), obtained from the genebank of the National Biotechnology Information Center (NCBI), was used. [Https://www.ncbi.nlm.nih.gov/nuccore/AF492815.1]

The ApE v2.0.47 (A Plasmid Editor) program was used to modify the coding sequence (ORFs determination, introns removal, choice of enzymes and primer construction), which is a free-access program that allows the visualization of the coding region translation and identification and display of restriction enzyme sites within the DNA. Subsequently, the presence of some signal peptide or transmembrane regions in the gene was analyzed to avoid its presence in the sequence. To do this, the tool we used was "Signal Blast" of C.A.M.E. (Center of Applied Molecular Engineering) [http://sigpep.services.came.sbg.ac.at/signalblast.html].

Once the sequence of the gene of interest was modified, restriction enzyme recognition sequences present in the polylinker of each vector were placed at the ends of the gen, with the condition that they don't cut into any part of the gene sequence. The restriction sites were inserted using the Enzyme Selector function of the APE software.

Finally, it was verified that the inserted sites did not run in the reading frame and when it finished, the final sequence was generated in FASTA format.

Design of cloning vector

The cloning vector was designed from the plasmid pUC18 (Figure 1), which is characterized by having many cloning sites and a selection gene (ampicillin resistance).

The sequence of the plasmid was obtained from the SnapGene bank [http://www.snapgene.com/resources/plasmid_files/basic_cloning_vectors/pUC18/] and using the ApE and SnapGene programs we analyzed the enzymes to be used for insertion of the CynD gene in the plasmid pUC18 and the sequences of the primers. Then, the CynD sequence was inserted *in silico* in the plasmid pUC18, thereby obtaining the cloning plasmid.

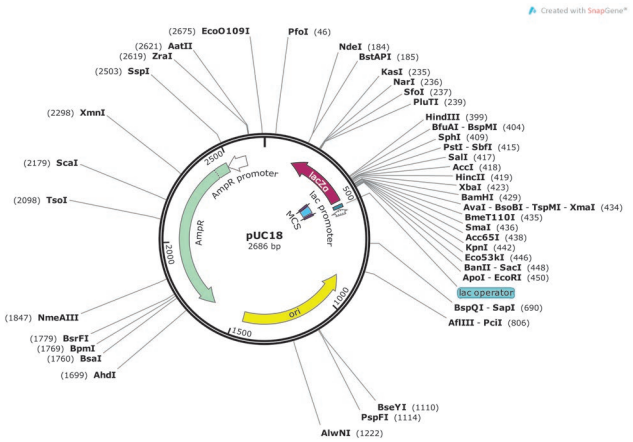

Figure 1. Plasmid pUC18 obtained from SnapGene.

Design of transformation vector for *A. tumefaciens.*

A. tumefacens is used as a vehicle for the genetic transformation of plants. To do this, the bacterium with its intact Ti plasmid is used as the vector, without disassembly and another plasmid is inserted where the gene of interest between T-DNA borders is found.

In this case, the transformation vector was designed from the plasmid pBI121 (Figure 2), widely used for the transformation of plants. For this, the sequence of the plasmid was obtained in the SnapGene bank [http://www.snapgene.com/resources/plasmid_files/plant_vectors/pBI121/] and we worked on the Ape v2.0.47 program for the insertion of the gene Interest therein by performing a simulation of the cut of the plasmid at the confined site and a subsequent ligation of the gene sequence prepared previously. The result was displayed in the SnapGene® Viewer V 2.8.2 software.

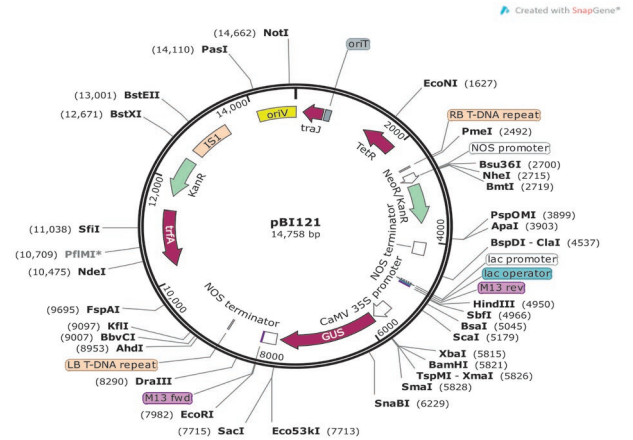

Figure 2. Plasmid pBI121 obtained from SnapGene.

RESULTS

Modification of the coding sequence

The CynD gene sequence was obtained from the NCBI gene bank (AF492815) and its ORF was determined between 134 and 1192 nucleotides, the remainder of the sequence was deleted. No signal peptides or transmembrane regions were identified in the sequence, using the Signal Blast tool of C.A.M.E.

For the insertion of the CynD into the cloning vector (plasmid pUC18), restriction enzyme recognition sequences, BamHI (G'GATCC) and EcoRI (G'AATTC) were placed at the gene ends, and the forward primers and reverse were designed. (Table 1).

Table 1. Primers constructed to insert CynD into plasmid pUC18.

Primers sequences for inserting CynD into plasmid pUC18	
Primer Forward	AACGGATCCATGTCATCCAAACTTCATATTTCCT
Primer Reverse	TGGTATACTGGAAGAAAAAGTTTAAGAATTCCCA

For the insertion of CynD into the transformation vector (plasmid pBI121) restriction enzyme recognition sequences, ScaI (AGT'ACT) and BamHI (G'GATCC), were placed at the gene ends, and the forward primers and reverse were designed (Table 2).

Table 2. Primers constructed to insert CynD into plasmid pBI121.

Primer sequences for inserting CynD into plasmid pBI121	
Primer Forward	AAAAGTACTATGTCATCCAAACTTCATATTTCCT
Primer Reverse	TGGTATACTGGAAGAAAAAGTTTAAGGATCCCAA

Design of cloning vector

With the ApE v2.0.47 software, the CynD gene was inserted into the polylinker of plasmid pUC18 between the EcoRi (5'GAATTC - 3'CTTAAG) and BamHi (5'GGATCC - 3 'CCTAGG) enzymes of the multiple cloning site.

The plasmid created: pUC18-CynD (Figure 3) also contains an origin of replication encoding for the initiation of DNA synthesis, an ampicillin resistance gene as a selection agent and a bacterial lacZ gene fragment as a metabolic marker (Louro & Crichton, 2013).

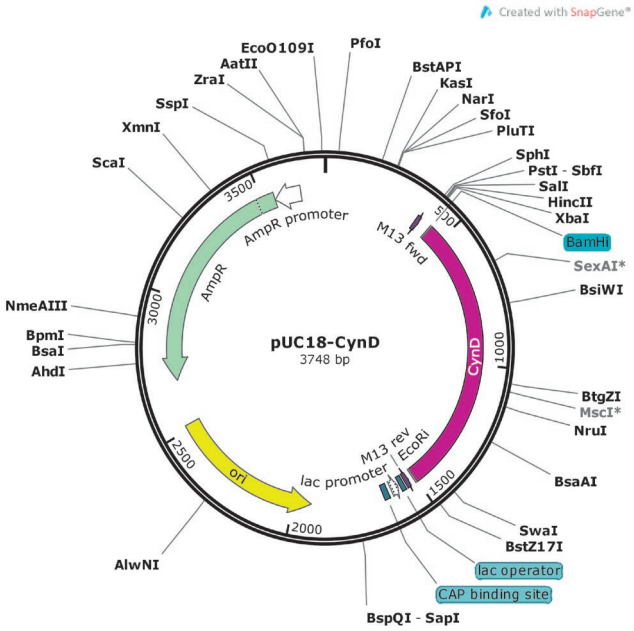

Figure 3. SnapGene diagram of cloning vector in after insertion of CynD.

Design of transformation vector for *A. tumefaciens*

With the ApE v2.0.47 software, the CynD gene was inserted into the polylinker of the plasmid pBI121, between ScaI (5'GAGCTC - 3'CTCGAG) and BamHI (5'GGATCC - 3'CCTAGG) enzymes.

The plasmid created: pBI121-CynD (Figure 4), also contains a gene encoding the enzyme β-glucuronidase from *E. coli* (GUS), a 35S promoter from the cauliflower mosaic virus (35SCaMV) and the NPTII gene Neomycin phosphotransferase II, which confer resistance to kanamycin in plants.

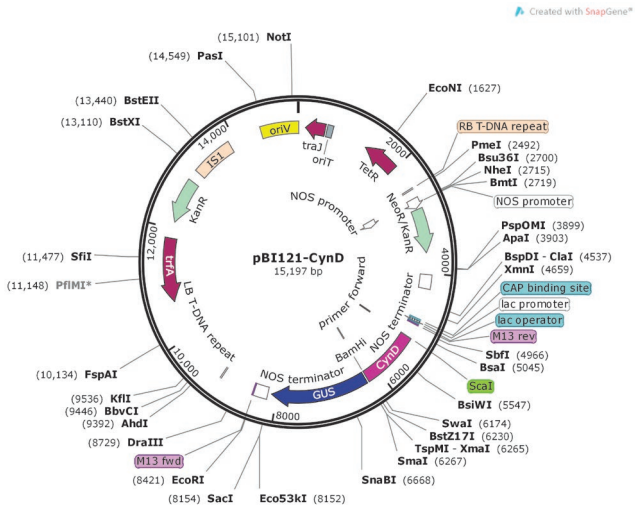

Figure 4. SnapGene diagram of the transformation vector after insertion of CynD.

DISCUSSION

The enzymes nitrilase, nitrilohydrate, cyanide hydrate (CHT) and cyanide dihydratase or cyanidase (CynD) are responsible for the degradation of CN by microorganisms (Ebbs, 2010). Of these, cyanide dihydrates readily convert cyanide into relatively non-toxic products (formate and ammonia) and does not require cofactors. So the gene encoding this enzyme, CynD, from *B. pumilu*s C1, has been cloned, sequenced and being used in numerous genetic engineering research.

In other studies, it was constructed vectors containing CynD for the heterologous expression of these enzymes in *A. thaliana* (Logan & Leaver, 2000). To do this, it was used different plasmids compatible with the Gateway cloning technology as the pENTRTMTOPO® vector to clone the effector and a target vector pFAST, which directs the constitutive expression of the gene of interest under the control of the CaMV 35S promoter (Shimada *et al*., 2010). In contrast, in this project, we have designed two plasmids compatible with traditional digestion technology by restriction enzymes and ligases. As a cloning vector, pUC18 was used as the base, which contains a bacterial lacZ gene fragment that allows the simple identification of the recombinant plasmids since it produces blue colonies and if a DNA fragment is inserted into the polylinker, this gene is inactivated giving rise to white colonies. And as a transformation vector, pBI121, that contains a gene encoding the enzyme β-glucuronidase from *E. coli* (GUS), a CaMV 35M promoter and the NPTII gene (neomycin phosphotransferase II) conferring resistance to Kanamycin in plants.

For the design of plasmids and constructs, there are several programs and web platforms such as Gene Design 3.0 (Richardson *et al.,* 2010), Gene Designer 2.0 (Villalobos *et al.*, 2006), GeMS (Jayaraj *et al.,* 2005), Bioedit 7.2.5 (Hall, 1999), Visual Gene Developer 1.3 (Jung & McDonald, 2011), among others. In this project we used the programs SnapGene and APE v2.0.47 (A Plasmid Editor), due to the tools that they offer for the visualization of the region of coding and identification of restriction enzyme sites inside the plasmid and for being programs of easy access.

It was possible to design two types of plasmids that would allow the transfer of the gene CynD to *Arabidopsis thaliana* via *A. tumefaciens*, achieving the introduction of a metabolic pathway of synthetic cyanide degradation from *Bacillus pumilu*s, thus allowing to increase the tolerance of the plant to CN and with it, greater phytoremediation options.

The cloning vector (pUCCynD) was designed by inserting in silico the CynD sequence in the polylinker of the plasmid pUC18, between the EcoRi (5'GAATTC-3'CTTAAG) and BamHi (5'GGATCC-3'CCTAGG) enzymes of the multiple cloning site.

The transformation vector (pBCynD) was designed by inserting in silico the CynD sequence in the polylinker of the plasmid pBI121, between the enzymes ScaI (5'GAGCTC - 3'CTCGAG) and BamHI (5'GGATCC - 3'CCTAGG).

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