



Research Article

Cloning and Heterologous Expression of Cyanobacterial Genes Encoding Phosphinothricin N- acetyltransferase in *E. coli*

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Abstract: Phosphinothricin N-acetyltransferase (Pat) is a bacterial enzyme that is introduced into plants to confer resistance to herbicides containing phosphinothricin (PPT). The expression of the Pat protein in transgenic plants allows them to tolerate the herbicide glufosinate. Although the enzyme has been identified in cyanobacteria through sequence analysis, its activities have not yet been thoroughly investigated. In this study, we cloned the putative Pat enzyme-encoding genes *alr4468* and *sll1647* from *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803, respectively, into the His-Tagged pET28a+ expression vector. The recombinant vectors containing these genes were introduced into protease-free *E. coli* BL21 cells. We successfully expressed the Alr4468 and Sll1647 polypeptides in the recombinant *E. coli* BL21 cultures that were induced with 1 mM IPTG. The highest in vitro Pat activities were measured using enzyme assays from IPTG-induced cell crude extracts, showing increases of 3.47 times for rAlr4468 and 2.53 times for rSll1647 compared to controls. As a result, the *alr4468* and *sll1647* genes from cyanobacteria, which exhibit similar physiological properties to those of high-structured plants, may serve as potential sources for developing herbicide-resistant transgenic plants in future research.

Keywords: *bar* gene, Cyanobacterial *pat* gene, Herbicide resistance, Phosphinothricin N-acetyltransferase

Fosfinotrisin N-asetiltransferazı Kodlayan Siyanobakteriyel Genlerin *E. coli*'de Klonlanması ve Heterolog İfadesi

Öz: Fosfinotrisin N-asetiltransferaz (Pat), fosfinotrisin (PPT) içeren herbisitlere direnç sağlamak için bitkilerde aktarılan bakteriyel bir enzimdir. Pat proteininin transgenik bitkilerde ifade edilmesi, glufosinat herbisitine karşı tolerans sağlar. Siyanobakterilerdeki bu enzim dizi analizi ile tanımlanmış olmasına rağmen, aktiviteleri henüz belirlenmemiştir. Bu çalışmada, *Anabaena* sp. PCC7120 ve *Synechocystis* sp. PCC6803'de Pat enzimini kodladığı varsayılan sırasıyla *alr* ve *sll* genleri His-Taq pET28a+ ekspresyon vektörüne klonlandı. İlgili genleri içeren rekombinant vektörler proteaz içermeyen *E. coli* BL21 hücrelerine transfer edildi. Alr4468 ve Sll1647 polipeptitleri 1 mM IPTG ile indüklenmiş rekombinant *E. coli* BL21 kültürlerinde başarıyla ifade edildi. En yüksek *in vitro* Pat aktiviteleri IPTG ile indüklenmiş kaba hücre ekstraktları kullanılarak yapılan enzim deneylerinde elde edilmiş ve kontrollerden sırasıyla 3.47 (rAlr4468 için) ve 2.53 (rSll1647 için) kat daha yüksek bulunmuştur. Sonuç olarak, yüksek yapılı bitkilerle benzer fizyolojik özelliklere sahip olan siyanobakterilerin *alr4468* ve *sll1647* genleri, gelecekteki çalışmalarda herbisite dirençli yerli transgenik bitkilerin üretimi için potansiyel kaynaklar olarak kullanılabilirler.

Anahtar Kelimeler: *bar* geni, Fosfinotrisin N-asetiltransferaz, Herbisit direnci, Siyanobakteriyel *pat* geni

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1. Introduction

Weeds, insects, and pathogens are among the most important factors that decrease agricultural productivity. Reports show that weeds reduce agricultural productivity about 34% by competing for nutrients with the plant to be grown (Oerke, 2006). Therefore, herbicides are intensively used in agriculture to minimize crop loss. Phosphinothricin (PPT), also known as Glufosinate, is a natural antibiotic that was initially isolated from *Streptomyces viridochromogenes* (Bayer et al., 1972). The ammonium salt of PPT, known as glufosinate ammonium, has been used as a highly effective, broad-spectrum herbicide for weed control under the trade name Basta since 1980. PPT is an analog of glutamic acid and competes with this amino acid by binding to glutamine synthetase (GS), thus inhibiting nitrogen assimilation. When GS is inhibited in plant cells, it leads to a deficiency of glutamine and an accumulation of ammonia. This accumulation of ammonium in tissues is known to suppress photosynthesis (Wild & Manderschild, 1984) and negatively affect photophosphorylation (Vencill, 2002).

Recent studies indicate that PPT primarily exerts its phytotoxic effects by increasing the levels of reactive oxygen species (ROS) within plant cells. This rise in ROS leads to the rapid peroxidation of lipids in the cell membrane, ultimately resulting in the cell death (Takano et al., 2020).

Streptomyces hygroscopicus and *S. viridochromogenes* also produce bialaphos-resistance (*bar*) (Thompson et al., 1987) and phosphinothricin N-acetyltransferase (*pat*) enzymes (Wolleben, et al., 1988), respectively, that confer resistance to phosphinothricin. These enzymes detoxify PPT by adding an acetyl group to its free amino group (Murakami et al., 1986), and two genes have been successfully utilized to engineer commercial transgenic crops. Additionally, the PPT resistance gene serves as a crucial tool in plant biotechnology, acting as a selectable marker (Miki & McHugh, 2004). PPT has widespread applications in South America and Asia, and it is also commonly used in regions where glufosinate-resistant soybeans and cotton are cultivated, particularly in North America. Although the herbicide was registered for use in Europe until 2018, it was withdrawn by the European Commission due to concerns about its toxicity (Takano & Dayan, 2020). Nevertheless, it is anticipated that the ban on paraquat in many major agricultural countries will significantly increase the demand for glufosinate (Donthi & Dileep Kumar, 2022). For this reason, resistance to glufosinate in plants achieved through the transfer of these genes. Plants in which the *bar* or *pat* gene has been transferred include cotton (Keller et al., 1997), lettuce (Mohapatra et al., 1999), sugar cane (Falco et al., 2000) and beans (Aragao et al., 2002).

Carbonari et al. (2016) developed LibertyLink® cotton cultivars resistant to glufosinate by overexpressing the *bar* gene. Insecticide-resistant WideStrike® cotton cultivars were created by using the *pat* gene as a selectable marker. In 2006, the Food and Drug Administration (FDA) of the United States approved the commercial production of glyphosate-resistant maize, cotton, sugar beet, canola and soybean (Mulwa & Mwanza, 2006). While *bar* and *pat* genes have been utilized for PPT resistance, research identifying sequences of genes that encode Pat and its activity in various organisms has been reported (Páez-Espino et al., 2015). The DNA sequences encoding the Pat enzyme have been identified in the cyanobacterial strains *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803. However, no study has yet reported the activity of this enzyme. In this study, *alr4468* and *sll1647* genes (Kaneko et al., 1996; Kaneko et al., 2001) encoding putative Pat enzyme from *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803, respectively, were amplified, cloned and expressed. The genes were amplified by PCR and ligated into the pGEM-T Easy cloning vector. Then, both gene regions were inserted into the pET28a+ expression vector. After expression of the recombinant proteins in *Escherichia coli* BL21, their potential Pat activities were analysed.

2. Material and Methods

2.1 Bacterial strains and growth conditions

E. coli DH5α and *E. coli* BL21 strains were grown in accordance with standard procedure. 50 µg/ml ampicillin or kanamycin was added to medium for selection. Inoue method was used to prepare competent *E. coli* cells (Sambrook & Russell, 2001). *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC7120 strains were grown in BG11 and BG110 medium, respectively (Rippka et al., 1979).

Cyanobacterial liquid cultures were incubated at 28°C, in 40 µmol/photon m⁻²s⁻¹, light intensity at 90 rpm. Bacterial strains and plasmids used in this study are given at Table 1.

Table 1. Bacterial strains and plasmids used in this study

Bacterial Strains and Plasmids	Definition	Reference
<i>Synechocystis</i> sp. PCC 6803	WT	Rippka et al., 1979
<i>Anabaena</i> sp. PCC 7120	WT	Rippka et al., 1979
<i>E. coli</i> DH5α	<i>F</i> ⁻ Φ80 <i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA</i> ⁻ <i>argF</i>) <i>U169 recA1 endA1 hsdR17</i> (<i>rK</i> ⁻ , <i>mK</i> ⁺) <i>phoA supE44 λ</i> ⁻ <i>thi-1 gyrA96 relA</i>	Sambrook & Russell, 2001
<i>E. coli</i> BL21	<i>fhuA2 [lon] ompTgal [dcm] ΔhsdS (NEB)</i>	Sambrook & Russell, 2001
<i>E. coli</i> GSLL	<i>E. coli</i> DH5α containing pGSLL	This study
<i>E. coli</i> GALR	<i>E. coli</i> DH5α containing pGALR	This study
<i>E. coli</i> ESLL	<i>E. coli</i> DH5α containing pESLL	This study
<i>E. coli</i> EALR	<i>E. coli</i> DH5α containing pEALR	This study
<i>E. coli</i> ESLL-BL	<i>E. coli</i> BL21containing pESLL	This study
<i>E. coli</i> EALR-BL	<i>E. coli</i> BL21containing pEALR	This study
pGSLL	pGEM-T Easy containing <i>Synechocystis</i> sp. PCC6803 <i>sll</i> gene	This study
pGALR	pGEM-T Easy containing <i>Anabaenas</i> p. PCC7120 <i>alr</i> gene	This study
pESLL	pET28+ with the <i>Synechocystis</i> sp. PCC6803 <i>sll</i> gene ligated to the <i>EcoRI</i> site	This study
pEALR	pET28+ with the <i>Anabaena</i> sp. PCC7120 <i>alr</i> gene ligated to the <i>EcoRI</i> site	This study
pGEM-T easy vector	Ap ^r , <i>lacZ'</i>	Promega Company
pET28a(+) expression vector	Km ^r , N-terminal His Tag	Novagene Company

2.2. Isolation of genomic and plasmid DNA

Genomic DNA isolation from cyanobacterial strains was performed according to Lind et al. (1985). Plasmid DNA isolation from *E. coli* was carried out with a Gene Jet plasmid DNA isolation kit (Thermo Fisher Scientific).

2.3. PCR conditions and primers

pat gene sequence of cyanobacterial strains were obtained from cyanobase database (Kaneko et al., 1996; Kaneko et al., 2001). For the amplification of the *pat* gene from the cyanobacterial strains *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803, specific primers alr4468F and alr4468R, sll1647F and sll1647R (Table 2) were designed using Primer3 (Untergasser et al., 2012), respectively. Adding *EcoRI* sequences to the 5' ends of the Forward (F) primers was made to guarantee that the reading frame would remain intact when transferred into the pET expression vector. *alr* and *sll* genes were amplified by PCR using *Pfu* DNA polymerase (Promega). PCR reactions were prepared in a total volume of 25 µl, consisting of the following final concentrations: 50 ng template DNA, 0.1 µM of forward (F) and reverse (R) primers, 200 µM dNTPs, 1.5 mM MgSO₄, 1x PCR buffer, and 2.5 units of *Pfu* DNA polymerase. The PCR conditions included an initial denaturation at 95°C for 2 minutes, followed by 25 cycles of denaturation at 95°C for 20 seconds, annealing at 61°C for 20 seconds (for the *alr* gene), and 64°C for 20 seconds (for the *sll* gene), with an elongation step at 68°C for 30 seconds. Finally, an elongation step of 5 minutes at 68°C was performed.

PCR amplification was conducted using Q5® High-Fidelity DNA Polymerase (NEB) to verify the obtained *alr* and *sll* clones. A total volume of 25 µl was prepared in PCR tubes, which contained the following final concentrations: 50 ng of template DNA, 0.2 µM of F and R primers, 200 µM of dNTPs, 2.5 units of Q5® High-Fidelity DNA Polymerase, and 1x PCR buffer. The PCR conditions was determined according to the procedure described by manufacturer.

Table 2. Primers used for amplification of *pat* genes (underlined region indicates *EcoRI* recognition sequence)

Primers	Sequences	T _m
sll1647F	5' CTGAATTCGTGATCCCATCTGACCTTTTC 3'	64°C
sll1647R	5' CTCCTAGGTCATTGTGAGGTTGTTAACCGTA 3'	64°C
alr4468F	5' CTGAATTCGTGATGACCATCCGTCAT 3'	61°C
alr4468R	5' CACCTAGCCTAAATTCCTTAATCCCATAATTAC 3'	61°C

2.4. Cloning procedure

Restriction analysis, ligation, agarose gel electrophoresis and transformation were carried out in accordance with standard molecular biology techniques (Sambrook & Russell, 2001). A GeneJet gel extraction kit (Thermo Fisher Scientific) was used to isolate DNA from agarose gel. The DNA concentration was measured using a Nanodrop ND-2000 (Thermo Fisher Scientific). The genes *alr4468* and *sll1647* were amplified via PCR using *Pfu* DNA polymerase, resulting in PCR amplicons of 496 bp and 512 bp, respectively. A PCR clean-up kit (ThermoFisher Scientific) was employed to purify the PCR amplicons. To add a tail to the 3' end of the amplicons, *Taq* DNA polymerase was utilized, as *Pfu* DNA polymerase generates blunt ends, and purification was performed again. The *pat* amplicons were individually ligated into the pGEM-T Easy vector (Promega Corp.) using T4 DNA ligase (Promega). The ligation mixtures were then transferred into *E. coli* DH5α competent cells. For positive selection of transformants, 50 µg/mL of ampicillin was added. Each of resulting plasmid was named, and each of clone was assigned a strain name (see Table 1). DNA sequencing of each cloned fragment was performed by Macrogen (Europe), which utilized Sanger sequencing with an Applied Biosystems 3730XL sequencer.

The *sll1647* and *alr4468* genes separately inserted into the pGEM-T easy vector were isolated from the plasmid by *EcoRI* restriction endonuclease treatment. They were ligated separately into a pET28+ vector with T4 DNA ligase following the conditions recommended by the manufacturer. The constructed plasmids and their clones were each given unique names. Their virtual maps were drawn with pDRAW32 software (AcaClone Software; <http://www.acaclone.com>). These plasmids were introduced into competent *E. coli* BL21 cells. To select the transformants positively, SOB medium containing 30 µg/mL of kanamycin was used. The *E. coli* cells harboring the related plasmids were named as listed in Table 1.

2.5. Purification of Pat proteins

Preparation of cell-extract from recombinant *E. coli* cells induced by IPTG was carried out in accordance with Şafak et al (2020). To His-Taq purification of Pat proteins, column (Macherey-NagelProtino® Ni-TED 2000) was equilibrated with 4 µl LEW buffer (also it contains 10 mM imidazole pH:8.0), and supernatant was applied slowly. The column was washed three times with LEW buffer including 20 mM imidazole. Then proteins were eluted with 3 ml elution buffer. 10 µl of that sample was separated to control of expression, and was used for SDS-PAGE analysis. The remaining eluent was renatured through dialysis (1 L, 200X volume of protein solution) at 4°C for 16 hours.

Determination of protein concentration was performed as described by Bradford (1976). Proteins were separated on polyacrylamide gels with 4.5% stacking and 16% separation under

denaturation conditions (Laemmi, 1970). SDS-polyacrylamide gels were stained with Coomassie Blue R-250, and were monitored after purification.

2.6. Pat enzyme activity experiments

Measurement of Pat enzyme activities from wild type cyanobacterial and recombinant *E. coli* strains was performed by as described Paez-Espino et al. (2015) and Thompson et al. (1987) with some modifications. The cultures were centrifuged and the pellets were used for protein extraction. PAT activity was determined by measuring the formation of 2-nitro-5-thiobenzoate (TNB) from 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and S-CoA spectrophotometrically at A_{412} after PAT catalysis. The reaction mixture was composed of 5 mM PPT, 1.23 mM acetyl coenzyme A (AcCoA), and Pat enzyme source (200 μ g of total protein). These components were prepared with deionized water (dH_2O) adjusted to a pH of 7.4, resulting in a total reaction volume of 350 μ l. The mixture was incubated for 5 minutes at room temperature, after which enzyme activity was measured using a spectrophotometer at an absorbance of 412 nm. The molar extinction coefficient of TNB was taken as $13600\text{ M}^{-1}\text{ cm}^{-1}$ (Vinnemier et al., 1995).

All assays were performed in triplicate, and Pat enzyme activities were statistically analyzed using one-way ANOVA followed by Bonferroni post hoc testing, conducted in GraphPad software (www.graphpad.com). Error bars in the graphs represent 95% confidence intervals. Statistical significance is indicated by p-values as follows: *** $p < 0.001$ and **** $p < 0.0001$.

2.7. Phylogenetic analysis of Sll1647 and Alr4468

Protein sequences of Pat enzymes 12 different species were retrieved using the BLAST program available at the NCBI server. Multiple sequence alignments were performed using Clustal Waligner in the MEGA12 software (Kumar et al., 2024). The phylogenetic tree was constructed using a Neighbor Joining algorithm with 1000 replicates of Bootstrap test. The amino acid sequence of Pat enzyme from *Streptomyces viridochromogenes* was used as the outgroup when constructing the phylogenetic tree.

3. Results

3.1. Construction of PESLL and PEALR plasmids to expression of cyanobacterial Pat proteins

Genomic DNA was isolated from the *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC7120 liquid cultures. *alr4468* and *sll1647* genes encoding Pat protein were amplified, and amplicons were separately ligated into pGEM-T easy vector with T4 DNA ligase. Plasmid DNA was isolated from colonies formed after transformation of the recombinant plasmids into *E. coli* DH5 α . The recognition site of *Eco*RI was located at the 5' end of the forward primers designed for gene amplifications. Therefore, the cloning was confirmed by cutting the plasmids with *Eco*RI enzyme. The expected *alr4468* (550 bp), *sll1647* (579 bp) and pGEM-T vector (3 kb) fragments were observed in agarose gel electrophoresis (Figure 1). Plasmid DNAs containing *alr4468* and *sll1647* fragments were named pGALR and pGSLL, respectively, and *E. coli* DH5 α cells containing the plasmids separately were named *E. coli* GALR and *E. coli* GSLL.

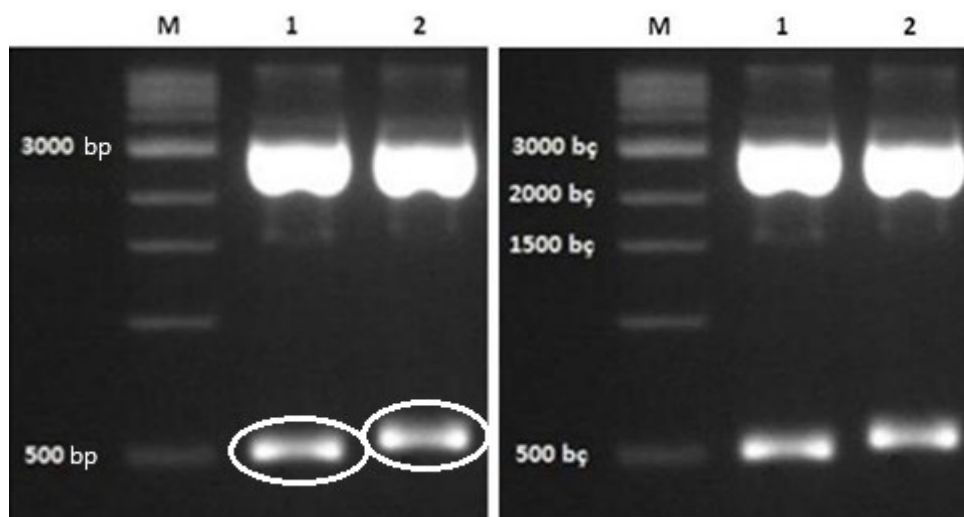


Figure 1. Agarose gel electrophoresis of *EcoRI* digestion of pGALR and PGSLL plasmids. M: 1 kb DNA Ladder (NEB3232), 1: *EcoRI*-cut pGALR, 2: *EcoRI*-cut PGSLL plasmid.

Pat genes cloned into the pGEM-T Easy vector were subcloned into the pET28a+ vector. The fragments containing the *alr4468* and *sll1647* genes, released by *EcoRI* digestion of the pGALR and pGSLL vectors, were ligated separately to the pET28a+ vector that was linearized using the same restriction enzyme. Then, ligation mixtures were transferred to *E. coli* DH5 α cells. Plasmid DNA was isolated from the colonies. The orientation of the *pat* gene, which is located into the multiple cloning site of the expression vector, is crucial for it to remain within the correct reading frame with the His-Tag. First, *pat* genes were randomly cloned into pET28+ vector within the *EcoRI* recognition site. Restriction fragment size analysis was then conducted to verify that the genes were inserted into the vector in the correct orientation. The *PvuI* recognition sequence is found at 4426 bp in the pET28a+ vector. The 373 bp of the *alr4468* gene also includes the cutting site for the same enzyme. Cutting the recombinant plasmid with *PvuI* yielded the expected 1492 bp fragment in the correct direction of entry (Figure 2a). The pET28a+ vector containing *alr4468* was named pEALR, and *E. coli* DH5 α cells carrying this plasmid were named *E. coli* EALR. The position of the *alr4468* gene in pEALR is given in "Figure 2b".

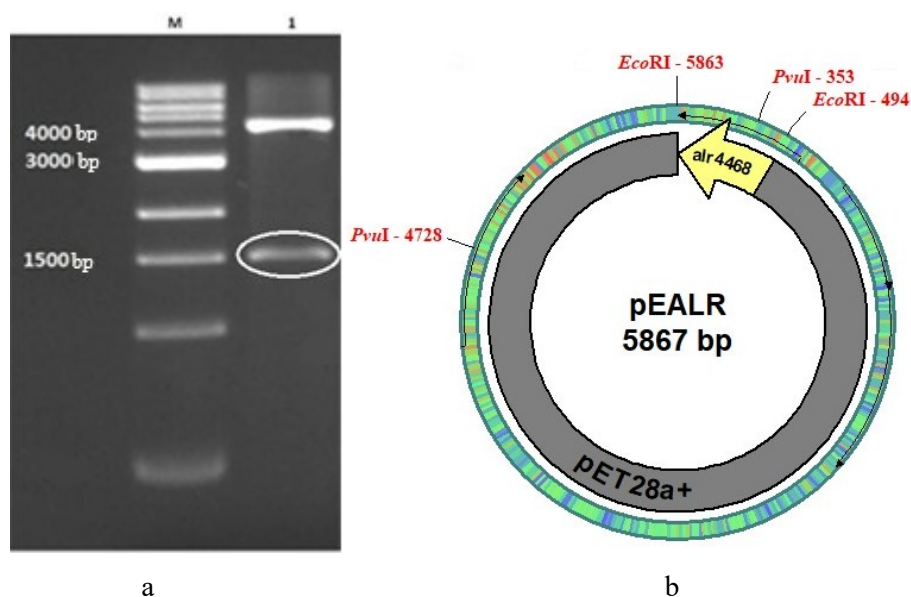


Figure 2. a) pEALR plasmid cutting with *PvuI*. M: 1 kb DNA Ladder (NEB3232), 2: pEALR cutting with *PvuI* b) Physical map of the plasmid pEALR.

The *HincII* restriction enzyme cuts the pET28a+ vector at 182 bp and 1630 bp positions. *sll1647* gene has a cutting site of the same enzyme that contains recognition sites at position 503 bp. The *sll1647* gene was inserted into the pET28a+ vector at the *EcoRI* site (192 bp). After cutting off the pET28a+ vector, containing the *sll1647* (579 bp) gene, with *HincII*, the expected 1941 bp fragment was obtained in the correct insertion direction (Figure 3a). The pET28a+ vector containing *sll1647* was named pESLL, and *E. coli* DH5 α cells carrying this plasmid were named *E. coli* ESLL. The position of the *sll1647* gene in pESLL is given in Figure 3b.

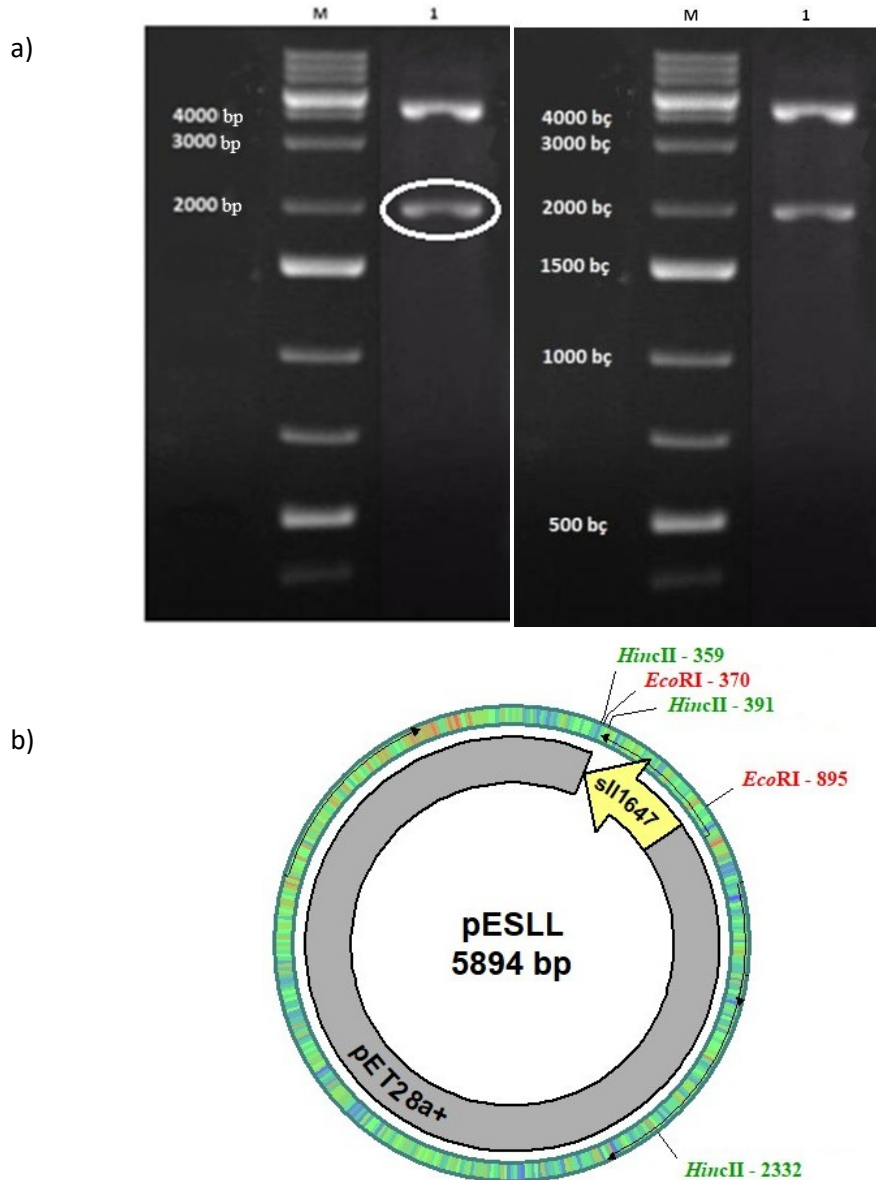


Figure 3. a) pESLL cutting with *HincII*. M: O'GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific) b) Physical map of the plasmid pESLL.

The pEALR and pESLL plasmids were isolated from *E. coli* DH5 α and transferred into *E. coli* BL21 cells separately. Clones were confirmed by PCR amplification to contain the correct insert and named *E. coli* EALR-BL and *E. coli* ESLL-BL.

3.2. Expression of *pat* genes in *E. coli* BL21 cells

The expression of rAlr4468 and rSll1647 proteins was achieved by induction of *E. coli* EALR-BL and ESLL-BL cells with 1 mM IPTG for 5 hours at 37°C. The expression of recombinant proteins in induced *E. coli* cultures was analyzed by SDS-PAGE (Figure 4).

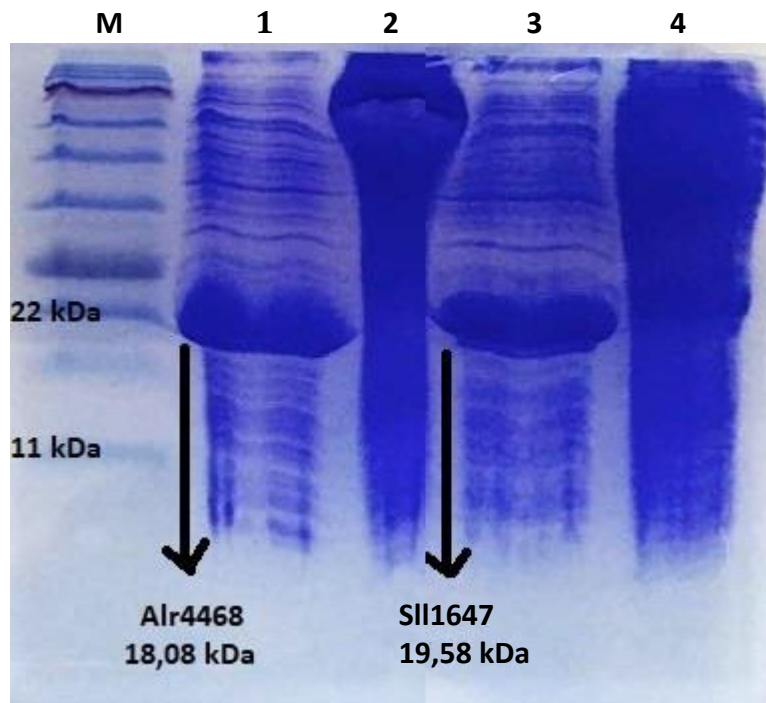


Figure 4. rAlr4468 and rAl1647 proteins in IPTG-induced *E. coli* EALR-BL and *E. coli* ESLL-BL extracts. M: Protein standards (NEB #P7712), 1: rAlr4468 expression in IPTG-induced *E. coli* EALR-BL, 2: Uninduced total protein sample of *E. coli* EALR-BL culture (Control), 3: rSll1647 expression in IPTG-induced *E. coli* ESLL-BL, 4: Not induced total protein sample of *E. coli* ESLL-BL culture (Control).

3.3. Purification of Pat proteins

Recombinant Pat proteins (rAlr4468 and rSll1647) were purified from *E. coli* strains EALR-BL and ESLLBL using a Protino® Ni-TED 2000 (Macharey Nagel) purification column. Expression of the recombinant proteins was analyzed by SDS-PAGE (Figures 5 and 6).

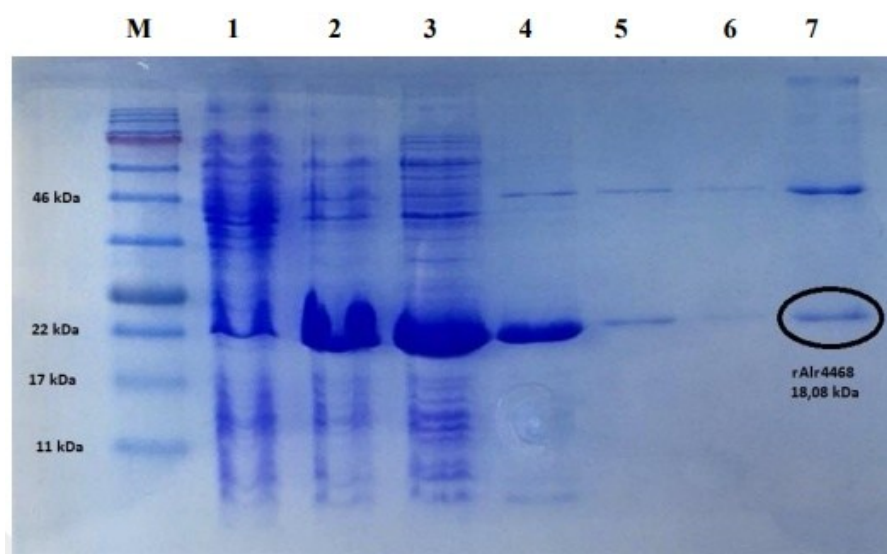


Figure 5. Purification of rAlr4468 protein. M: Protein standards (NEB #P7712), 1: Not-IPTG-induced *E. coli* EALR-BL total protein sample, 2: *E. coli* EALR-BL total protein induced with 1 mM IPTG, 3: Flow-through sample, 4: First wash of sample, 5: Second wash of sample, 6: Third wash of sample, 7: Eluent.

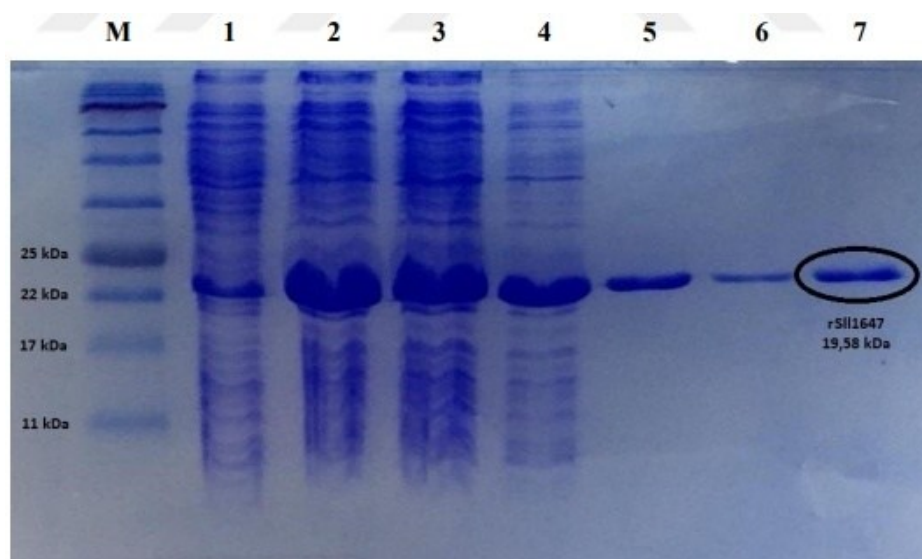


Figure 6. Purification of rSll1647 protein. M: Protein standards, 1: *E. coli* ESLL-BL total protein sample uninduced by IPTG, 2: *E. coli* ESLL-BL total protein induced with 1 mM IPTG, 3: Flow-through sample, 4: First wash of sample, 5: Second wash of sample, 6: Third wash of sample, 7: Eluent .

3.4. Determination of Pat enzyme activities in *E. coli* EALR-BL and ESLL-BL

To determine the Pat activities of the rAlr4468 and rSll1647 polypeptides, three different samples were used in the assay: (i) 40 µg of the purified rAlr4468 and rSll1647, (ii) The total protein samples (200 µg) obtained from IPTG-induced *E. coli* EALR-BL and ESLL-BL (ALR4468-IPTG and SLL1647- IPTG). The total protein samples from the IPTG-uninduced cultures of *E. coli* EALR-BL and ESLL-BL as the control groups (ALR4468-C, SLL1647-C). Distilled water served as the reaction buffer. The Pat enzyme activity results were shown in Figures 7 and 8.

To determine the Pat activities of the rAlr4468 and rSll1647 polypeptides, 40 µg of recombinant proteins (rAlr4468 and rSll1647) purified from IPTG-induced *E. coli* EALR-BL and ESLL-BL cell extracts were used. Control samples were prepared using cell extracts that were not

induced with IPTG. Distilled water served as the reaction buffer. The activity graphs for Pat are displayed in Figures 7 and Figure 8.

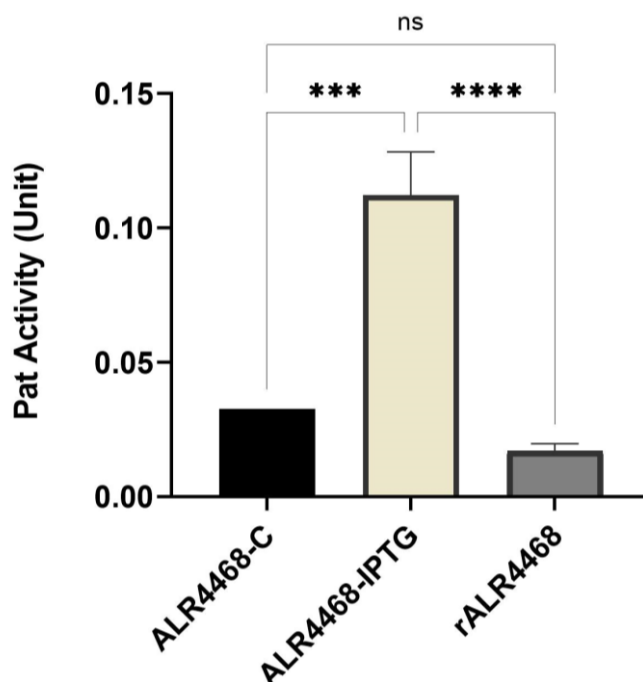


Figure 7. The activity of the Pat in *E. coli* EALR-BL strain under different conditions. The y-axis indicates Pat activity (in units), while the x-axis categorizes the data as follows: ALR4468-C an IPTG-uninduced control sample; ALR4468-IPTG, an IPTG-induced, and rALR4468 refers to the purified putative Pat enzyme obtained from cell induced with IPTG.

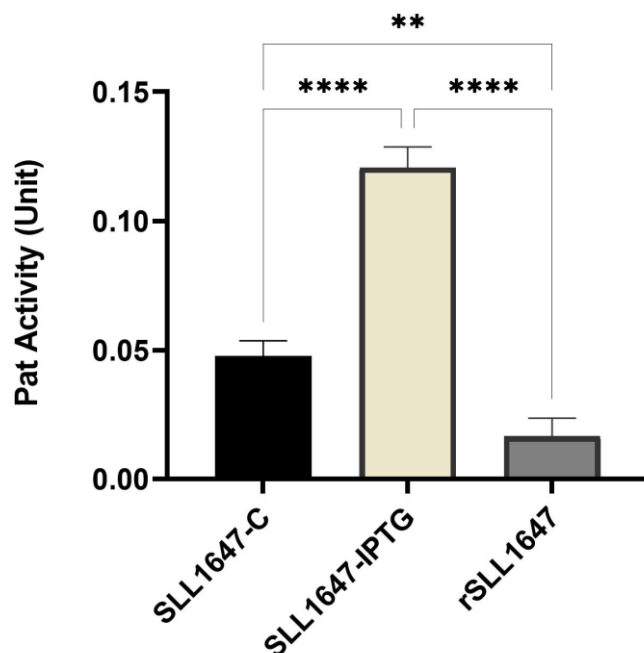


Figure 8. The activities of the Pat in *E. coli* ESLL-BL strain under different conditions. The y-axis indicates Pat activity (in units), while the x-axis categorizes the data as follows: SLL1647-C, an IPTG-uninduced control sample; SLL1647-IPTG, an IPTG-induced total protein sample, and rSLL1647 refers to the purified putative Pat enzyme obtained from cell induced with IPTG.

Accordingly, both the rAlr4468 and rSll1647 proteins were confirmed to exhibit Pat activities. rAlr4468 and rSll1647 proteins are present in the related cell extracts. IPTG-uninduced *E. coli* EALR-BL and *E. coli* ESLL-BL controls showed an aerge of 0.03 and 0.04 units/ml Pat activity, respectively. IPTG-induced *E. coli* EALR-BL and *E. coli* ESLL-BL cell extracts showed an average Pat activity of 0.11 and 0.12 units/ml, respectively. However, an average of 0.016 units/ml of Pat activity was detected in purified rAlr4468 and rSll1647 proteins.

3.5. Phylogenetic relationship of putative cyanobacterial Pat amino acid sequences

The amino acid sequences of the Alr4468 and Sll1647 proteins were compared with Pat enzymes from 12 different cyanobacterial species, and a phylogenetic tree was constructed, as shown in Figure 9. The amino acid sequence of Sll1647 exhibited 100% identity with a GNAT family N-acetyltransferase from an unclassified *Synechocystis* species. Similarly, the Alr4468 sequence showed 100% identity with the Alr4468 protein from *Anabaena* sp. PCC 7120 (= FACHB-418). When the two Pat proteins (Alr4468 and Sll1647) were compared, a sequence identity of 64.32% was observed, which explains their divergent placement in the constructed phylogenetic tree.

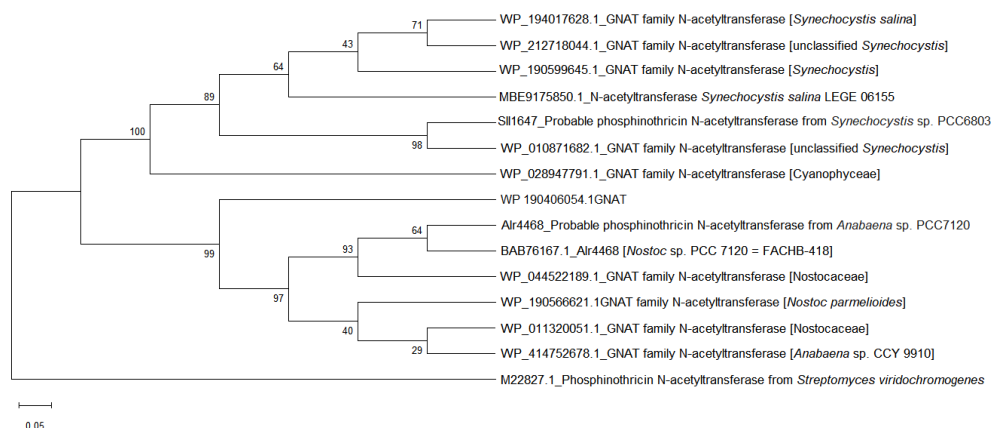


Figure 9. Phylogenetic dendrogram which shows amino acid sequences from 12 different cyanobacteria. Neighbour-Joining method with 1000 replicates of bootstrap test was used for constructing the phylogenetic tree. The *Streptomyces viridochromogenes* phosphinothricin N-acetyl transferase amino acid sequence (GenBank accession number: M22827.1) was used as the outgroup. The numbers in nodes refer to the Bootstrap value.

4. Discussion and Conclusion

Two glufosinate-resistant genes, *bar* and *pat*, were first isolated from *S. hygroscopicus* in 1987 and *S. viridochromogenes* in 1988, respectively. These genes are responsible for the acetylation of the amino group of L-PPT (Thompson et al.,1987; Wohlleben et al., 1988). Bartsch & Tebbe (1989) investigated PPT resistance in soil bacteria and found that PPT N-acetyltransferase, PPT oxidase, and PPT transaminase enzymes metabolize L-PPT differently. In that study, six out of eight soil bacteria exhibited PPT N-acetyltransferase activity. To date, no putative or identified genes encoding PPT transaminase and PPT oxidase have been reported in the Cyanobase database. The presence of genes encoding putative PPT N-acetyltransferase has been identified in 15 different cyanobacterial strains. However, there are no studies in the literature examining whether these genes exhibit functional polypeptides with Pat activity in the *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC7120 strains. Therefore, this study aimed to determine whether the putative *sll1674* and *alr4468* genes of *Synechocystis* sp. PCC6803 and *Anabaena* sp. PCC7120 exhibits PPT N-acetyltransferase activity.

The Alr4468 and Sll1647 proteins from *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803, respectively, were successfully expressed in IPTG-induced recombinant *E. coli* BL21 cells in this study. The cell extracts of IPTG-induced *E. coli* EALR-BL and *E. coli* ESLL-BL demonstrated Pat activities that were 3.47 times and 2.53 times higher, respectively, compared to the control

samples. In enzyme assays using the rAlr4468 and rSll1647 proteins, lower activity were observed. The activity of the Pat enzyme in IPTG-induced pEALR-BL and pESLL-BL cell extracts was 6.5-fold and 7.27-fold higher than the activities of the purified rAlr4468 and rSll1647 enzymes, respectively.

A comparison between other purified Pat enzyme activity and our results is difficult because we did not determine K_m -values for PPT. However, the low enzyme activity of purified recombinant proteins may be due to insufficient efficiency of transition to the active form in vitro or because the enzyme requires a component present in the cell extract, such as a cofactor. Additionally, in several cases where a comparative analysis of His-tagged and untagged protein preparations was conducted, differences between the two types of samples were observed. In some cases, the addition of a His-tag altered the structure of the target protein, while in other cases, it impacted the protein's functions (Kutyshenko et al., 2019). It can lead to the observation of lower acetylation activity in purified enzymes.

The development of transgenic plants that are resistant to herbicides containing phosphinothricin is crucial for enhancing agricultural yields. Research has shown that the *bar* and *pat* genes from *Streptomyces* sp., which belong to a distant physiological group, are transferred to plants (Yu et al., 2023; Li et al., 2023). Amino acid sequences of Pat and Bar proteins from *Streptomyces* sp. (Baker et al., 2000) and those of putative Pat proteins from cyanobacterial strains (Kaneko et al., 1996; Kaneko et al., 2001) were obtained from databases. Protein blast analysis using the Uniprot program (Coudert et al., 2023) revealed a 64.36% identity between the Alr4468 and Sll1647 proteins. There is a 27.78% and 28.28% protein identity between the Pat protein of *Streptomyces viridochromogenes* and the cyanobacterial Sll1647 and Alr4468 proteins, respectively. Furthermore, the alignment of amino acid sequences for Bar, Pat, and putative Pat polypeptides from cyanobacteria was performed using Megal2 software (Kumar et al., 2024). In all sequences was high conserved at Glu82, Tyr86, Gly95, Lue100, 124Asn and 127Ser, which are putatively to form a binding pocket for the substrate acetyl coenzyme A (Wu et al., 2014). However, the Ala118 position is conserved in Bar and Pat polipeptides from *Streptomyces* sp., whereas it is replaced by Gly in putative cyanobacterial Pat polypeptides. The two groups are phylogenetically distantly related. Use of *pat* from *S. viridochromogenes* as selectable marker usually resulted in the lowest level of transformation efficiency. It is known that optimizing the coding sequence with codons preferred by the target species can significantly enhance gene expression levels (Sivamani et al., 2019). It is important to note that Pat proteins isolated from different bacteria can exhibit varying kinetic constants and levels of glufosinate resistance across different cellular compartments or within plant cells. Therefore, conducting searches for new glufosinate-resistant genes is essential. Studies are being ongoing to determine the gene sequences encoding Pat and potential Pat activities in different organisms (Zhu et al., 2023). Cyanobacteria, like plants, possess a dual photosystem and are distinguished from other photosynthetic bacteria by this feature. Molecular phylogenetic studies have revealed that cyanobacteria are the closest bacterial relatives of plastids (Raven & Allen, 2003).

This study found that the putative Pat proteins from cyanobacteria exhibit PPT N-acetyltransferase activity. Using a gene derived from a photosynthetic organism is likely to enhance herbicide resistance in plants and boost crop yield. This study demonstrates that herbicide-resistant plant varieties can be developed by transferring these potential cyanobacterial genes, which share a closer physiological relationship with plants.

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