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PRODUCING A RECOMBINANT VECTOR THAT CARRIES THE VASCULAR CLASS III PEROXIDASE (*PRX1*) GENE FROM *Catharanthus roseus*

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ABSTRACT

Terpenoid indole alkaloids (TIAs) vincristine and vinblastine are two essential anticancer drugs used in cancer treatments. These compounds are naturally available only in *Catharanthus roseus* in low accumulation, leading to efforts to upregulate TIAs substances through other expression organisms. The combination of vindoline and catharanthine into α-3~,4~-anhydrovinblastine (AVLB) is an important step for the biosynthesis of vinca alkaloids, which serves as a precursor to vinblastine and vincristine. Vascular class III peroxidase (*PRX1*) plays a crucial role in catalyzing the dimerization reaction. In this study, we created a recombinant plasmid consisting of the *PRX1* gene with the aim to transform and express it stably in fungi. The full-length 1092 bp *C. roseus PRX1* coding sequence was optimized to be suitable for a fungal translation system while keeping its amino acid sequence unchanged. The pGreen3 vector was used as the backbone to harbor the *PRX1* and hygromycin-resistant gene (*HYG*)-which enabled fungi cells to survive in the HYG selection medium. This *HYG* gene was cut from the pGreen2 vector and inserted into the pGreen3 vector. Both *PRX1* and *HYG* genes are located between the gpdA promoter and TrpC terminator structure. The recombinant plasmids were screened and amplified by transformation into *Escherichia coli* DH10B, and cultured in the LB medium containing kanamycin antibiotic. Our recombinant vector is suitable for further expression into endophytic fungi.

Keywords: Anticancer drugs, *Catharanthus roseus*, *PRX1*, recombinant plasmid, vinblastine, vincristine.

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INTRODUCTION

Madagascar periwinkle (*Catharanthus roseus*) is a medicinal plant whose leaves are a unique source of two anticancer substances: vinblastine and vincristine. These major terpenoid indole alkaloids (TIAs) inhibit chromosome duplication and segregation, targeting the G2/M phase of mitosis at the cell cycle (McGrogan et al., 2008). Accordingly, they suppress cell growth and are used as anti-miotic drugs. These compounds have been used in the treatment of numerous cancer types, including Hodgkin's lymphoma, lung cancer, brain cancer, etc. (Caputi et al., 2018). Although the demand for these TIAs reaches 3 kg annually worldwide, their actual production from *C. roseus* is extremely low in quantities. It is estimated that 500 kg of dried leaves are required to obtain 1 g of these alkaloids (Balandrin & Klocke, 1988). This has led to research efforts to boost TIAs production through bioengineering strategies. One effective approach is stimulating their metabolism flux by upregulating their downstream gene activities. Therefore, the vincristine and vinblastine biosynthesis pathways and the enzymes involved are studied extensively.

The biosynthesis route of those vinca alkaloids relates to more than 20 enzymatic steps and several regulatory genes have been cloned (Costa et al., 2008). TIA regulation starts with the indole pathway and iridoid pathway at upstream stages then ends with the derivation of vinblastine and vincristine from α-3~,4~-anhydrovinblastine (AVLB). The vascular class III peroxidase (*PRX1*) is indicated as a key enzyme in this network with high metabolic plasticity as it couples the monomeric precursors' vindoline and catharanthine into the dimeric alkaloid AVLB (Sottomayor et al., 2008). Using gene-transform systems to express the *PRX1* gene in plants and fungi is a biotechnological approach of interest. This is a potential solution for enhancing enzyme activities and producing enough anticancer drugs.

The presence of natural endophytic fungi does not cause any disease in host plants (Kusari et al., 2012), they can promote plants' secondary metabolites biosynthesis or produce the same natural compound as their hosts (Kumar et al., 2013). The endophytic fungi community in *C. roseus* has been isolated and characterized in terms of their biodiversity, tissue specificity, and cytotoxic activity (Kharwar et al., 2008; Kuriakose et al., 2016; Lay et al., 2023). Among them, two strains of *Curvularia* sp. CATDLF5 and *Choanephora infundibulifera* CATDLF6 are able to increase the vindoline content - the precursor of vinblastine and vincristine by 229−403% via influencing the TIA biosynthesis genes and its regulating elements (Pandey et al., 2016). Researchers showed that fungi isolated from *C. roseus* as *Fusarium oxysporum* and *Talaromyces radicus* are capable of producing vinblastine and vincristine in culture media (Kumar et al., 2013; Palem et al., 2015; Tran Thi Huong Giang et al., 2021). These endophytes also upregulate the expression of *PRX1* to dimerize the vindoline and catharanthine to form AVLB (Costa et al., 2008; Pandey et al., 2016). However, after ligating a gene into the fungal genome, the effective secretory of heterologous proteins is generally low because of differences in the original source of the gene (Tanaka et al., 2014). One of the most efficient strategies to improve the production level of heterologous proteins in the host organisms is codon optimization (Gustafsson et al., 2004). In the study, a plasmid was designed to transfer the *C. roseus* in which *PRX1* gene with optimized codons to suitable with endophytic fungi in order to increase the expression level of this enzyme in fungi and plants.

MATERIALS AND METHODS

PRX1 sequence optimization and vector materials

The *PRX1* coding sequence (CDS) according to Costa et al. (2008) was revised by the GenSmart Codon Optimization online tool (GenScript Biotech Corporation, China). The nucleotide sequence was adjusted to be a high-performed generated sequence in fungi while its coding amino acids were unchanged.

This optimized sequence was ordered in the cloning vector pUC57 (pUC57+PRX1) (GenScript Biotech Corporation, China).

The pGreen2 vector that holds the hygromycin-resistant gene structure (*HYG*) and the pGreen3 vector that holds the Green fluorescent protein (*GFP*), and nourseothricin-resistant gene (*NAT*) marker structures were provided by the Key Laboratory of Enzyme and Protein Technology, University of Science, Vietnam National University, Hanoi. The 10.09 kb pGreen3 vector (Fig. 1) contained the kanamycin gene for bacterial selection and the structure from the Left Border (LB) to the Right Border (RB) consisting of *NAT* and *GFP* coding gene under the regulation of gpdA promoter region. Besides that, the pGreen2 structure includes an *HYG* gene, which was then transferred into the pGreen3 to replace the *NAT* gene. The competent *Escherichia coli* DH10B strain was kept at the Genomic Biodiversity Laboratory, Institute of Genome Research, Vietnam Academy of Science and Technology.

Inserting the *PRX1* gene into the plasmid vector

The pGreen3 vector was used as the backbone to harbor the revised *PRX1* gene sequence. Initially, pUC57+PRX1 and pGreen3 vectors were cut by *Bam*HI and *Sna*BI restriction enzymes (RE) (Thermo Fisher Scientific, Lithuania), releasing a 9.3 kb band for pGreen3 and a 1.1 kb band for *PRX1*. These electrophoresis gel bands were purified and ligated together by T4 ligase (Thermo Fisher Scientific, Lithuania). The ligation product pGreen3+PRX1 was transformed into *E. coli* DH10B by heat shock method. This strain was then cultured in a Luria-Bertani (LB) liquid medium containing kanamycin antibiotics. The extracted plasmids were cut by *Bam*HI and *Kpn*I (Thermo Fisher Scientific, Lithuania) to screen the recombined products.

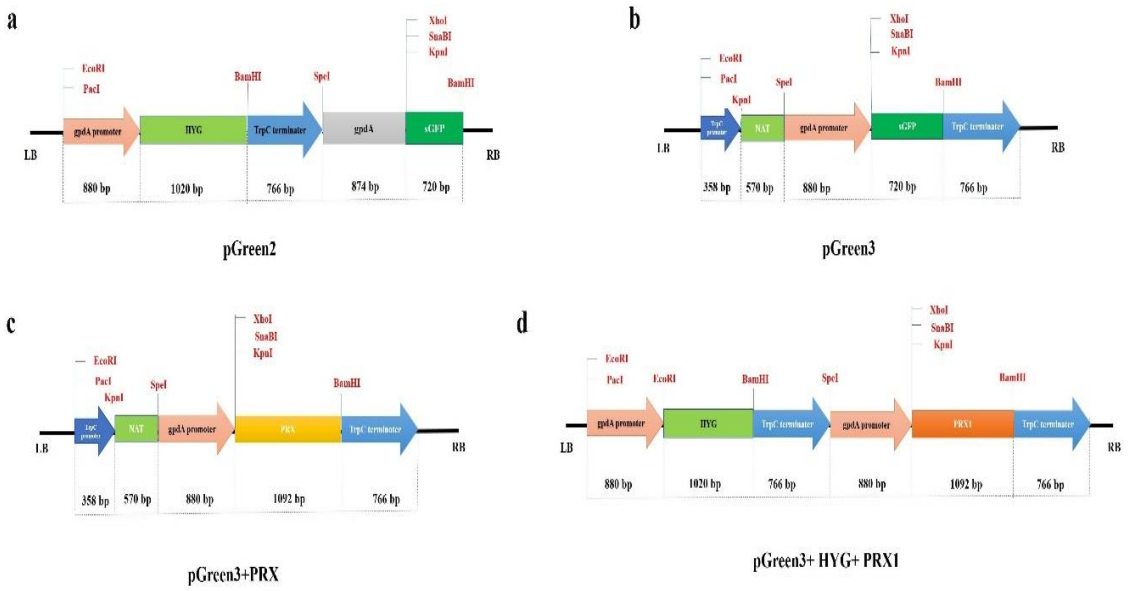

Figure 1. The map from the Left Border (LB) to the Right Border (RB) of the pGreen2 and pGreen3 vectors during the vector construction process

For transformation into endophytic fungi, these plasmids need to have hygromycin resistance ability, therefore, *HYG* was transferred from pGreen2 into the pGreen3+PRX1 vector. The procedure is performed similarly but using enzymes *Pac*I and *Spe*I (Thermo Fisher Scientific, Lithuania). The pGreen+PRX1+HYG recombinant vectors

were finally transformed into *E. coli* DH10B by heat shock method. Recombinant plasmids were screened in LB medium supplied kanamycin and then cut by *Bam*HI and *Kpn*I. The vector map from LB to RB of cloning vectors as illustrated in Figure 1.

RESULTS

PRX1 sequence optimization result and protein structure modeling

The *C. roseus PRX1* gene contains two intron regions, creating a 1092 bp mRNA after the transcription process. To optimize its coding sequence, 273 nucleotides out of a total of 1092 nucleotides were changed via the Codon Optimization tool (Fig. 2), which occupied 25% sequence length. Accordingly, GC% was increased from 43.32% in the original gene to 51.47%. The Codon Adaptation Index (CAI) of the gene was 0.80, which is considered a suitable rate to apply this sequence for the expression in fungi. The revised amino acid sequence was not affected so all the motif, conserved, and functional residues belonging to the plant class III peroxidase superfamily still remained. In the 363 amino acid sequence, the first 34 amino acids were N-terminal polypeptide (NTPP) signal - corresponding to the Endoplasmic reticulum signal peptide present in all plant peroxidase; followed by a mature PRX1 protein comprises approximately 304 amino acids; and then the C-terminal polypeptide extension with 23 to 25 amino acids. The secretory peroxidase conserved domain was found between 40th and 333th positions. As a member of the peroxidase superfamily, the gene contains four disulfide bridges established from eight conserved Cys (sites 16 and 97, 49 and 54, 103 and 298, 183 and 210), and two His residues interacting with the heme to catalyze multiple oxidative reactions involving hydrogen peroxidase (sites 38-AA GLLRLHF**H**DC and 168-DVVALSGG**H**TI) (Costa et al., 2008; Welinder, 1992).

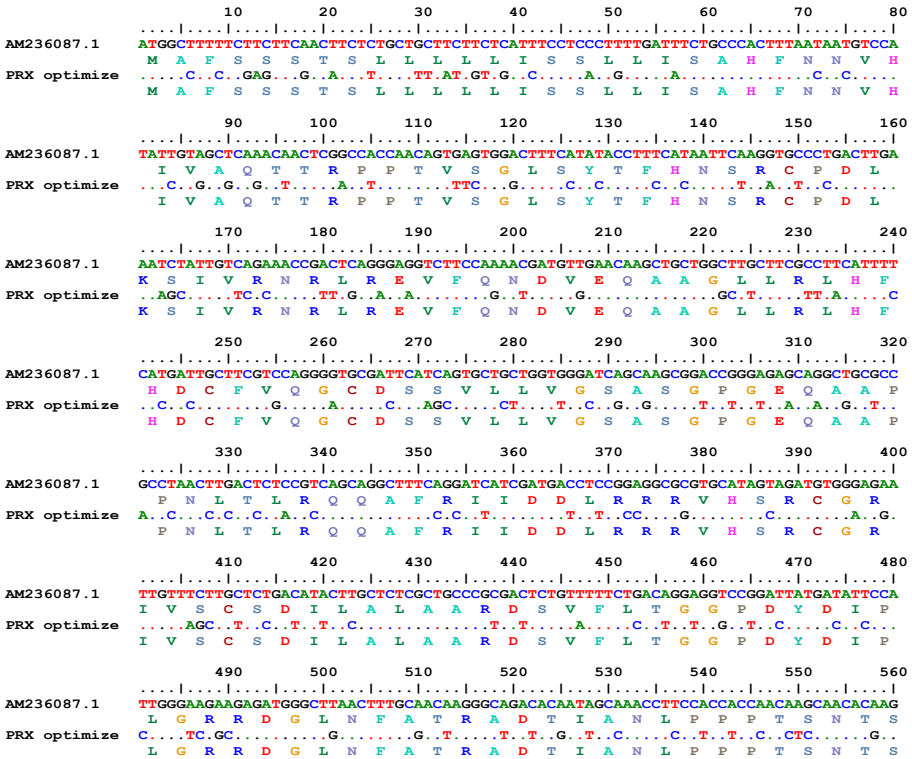

Figure 2. Alignment profile of the *PRX1* coding sequence in *Catharanthus roseus* before and after optimization

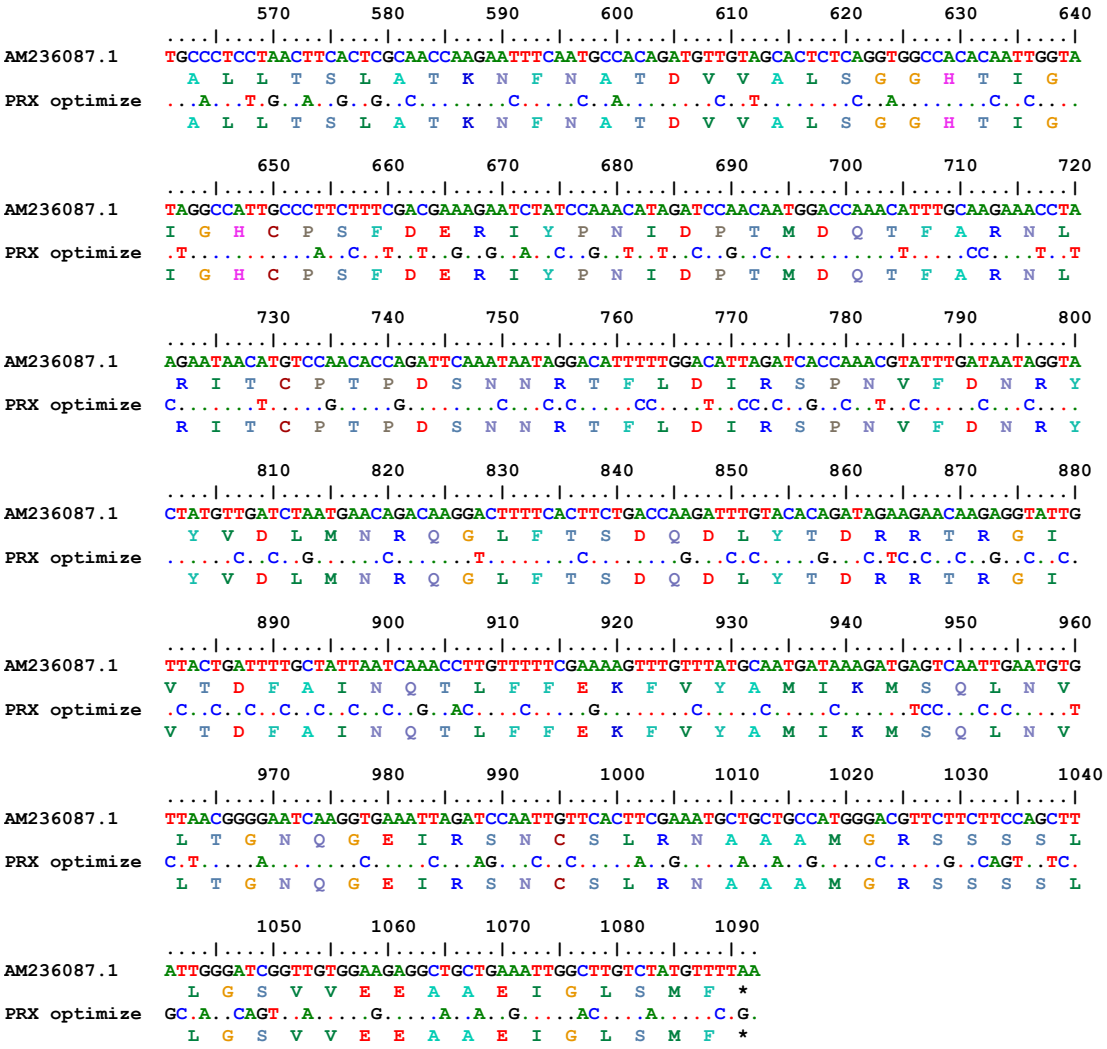

Figure 2. Alignment profile of the *PRX1* coding sequence in *Catharanthus roseus* before and after optimization (next)

A three-dimensional protein model of PRX1 was constructed based on its deduced amino acid sequence by SWISS-MODEL software (Waterhouse et al., 2028) (Fig. 3). Detailed information about the model, quality parameters, and template are indicated in Table 1. The PRX1 enzyme protein model was built based on deposit template A0A6J5Y0X4.1.A of *Prunus armeniaca* peroxidase via Alphafold 2 method (Jumper et al., 2021), which predicts PRX1's 3D structure from its amino acid sequence. The pairwise comparison between PRX1 and *P. armeniaca*'s peroxidase showed that the sequence identity and sequence similarity indices were 70.17% and 0.51% respectively. The *P. armeniaca*'s peroxidase reference sequence covered all of *PRX*'s amino acids with a 0.97 coverage index. The previous *CrPRX1* model was constructed based on the barley peroxidase 1 (*BP1*) (Costa et al., 2008) because of their close phylogenetic relationship, utilizing the X-ray crystallography method. However, the quality parameters inferred from *P. armeniaca* were higher than those from *BP1*, indicating higher reliability at 0.88 GMQE score (Table 1).

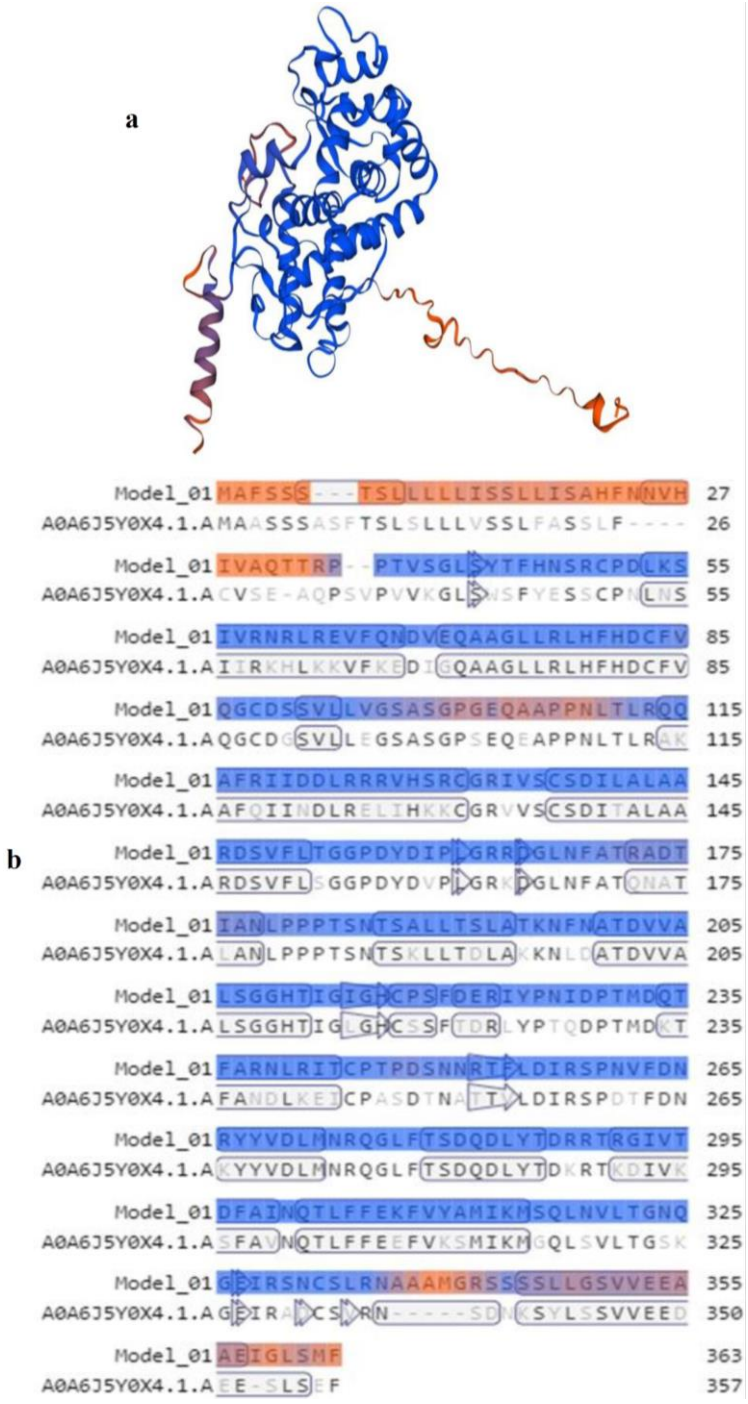

Figure 3. The three-dimensional structure of PRX1 protein (**a**) and its alignment result with the reference sequence from SWISS-MODEL (**b**). The model color is presented based on the QMEAN model quality: blue represents the highest reliability, with violet being lower and orange as the lowest. In the amino acid sequence alignment result, the rounded-border rectangles cover the alpha-helix fold sites, while the right-direction arrows indicate the beta-pleated sheet sites

Table 1. The constructed protein model quality parameters

Template	Description	Seq identity	Seq similarity	Oligo-State	Method	GMQE	Coverage (Range)
A0A6J5Y0X4.1.A	Peroxidase (*Prunus armeniaca*)	70.17%	0.51	Monomer	AlphaFold v2	0.88	0.97 (1–363)
1bgp.1.A	Peroxidase 1 (Barley grain)	57.28%	0.47	Monomer	X-ray, 1.90 Å	0.73	0.83 (35–363)

Vector construction examination

Several steps of inserting and cutting genes to build a pGreen3 vector harbor *PRX1* gene and hygromycin-resistant gene instead of a nourseothricin-resistant gene were done. The *PRX1* gene was inserted into the pGreen3 vector using the described method. The recombinant plasmid in *E. coli* DH10B's with heavier bands than the pGreen3's band on electrophoresis gel was isolated (Fig. 4). To validate these plasmids, we incubated them with *Bam*HI and *Kpn*I RE. Outcomes showed three bands 7.8 kb, 1.4 kb, and 1.1 kb which fit with the pGreen3 designed structure (there were two *Kpn*I cut sites in vector). This proved that a recombinant pGreen3+PRX1 vector has been successfully created, approximately 10.4 kb in length.

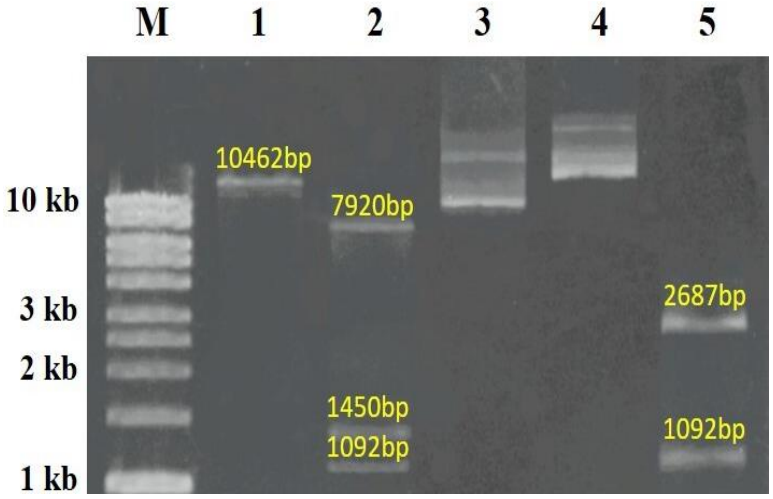

Figure 4. Validating the *PRX1* gene insertion result. M: Marker 1kb Bioline, 1: pGreen3+PRX1 cut by *Bam*HI, 2: pGreen3+PRX1 cut by *Bam*HI and *Kpn*I, 3: plasmid pGreen3, 4: plasmid pGreen3+PRX1, 5: plasmid pUC57+PRX1 cut by *Bam*HI and *Kpn*I

To replace the *NAT* gene from pGreen3+PRX1 with the *HYG* gene on pGreen2, these plasmids were cut by *Pac*I and *Spe*I RE. The backbone of pGreen3+PRX1 and the *HYG* gene were then ligated together. The recombinant plasmid was also verified by electrophoresis running on agarose gel after cutting by *Bam*HI and *Kpn*I RE (Fig. 5). The electrophoresis result presented three bands cut from pGreen3+HYG+PRX1 plasmid, being around 9.3 kb, 1.6 kb, and 1.1 kb (because of two *Bam*HI cut sites in vector). This plasmid was cut by *Bam*HI (well "2", Fig. 5) The lighter band approximately 2.8 kb, was consistent with the designed length illustrated in Figure 1d. The plasmids pGreen3+HYG+PRX1, pGreen3+PRX1, and original pGreen3 were also used as the control samples (Fig. 5), shows the similarity in size between

experimental and theoretical vectors. Accordingly, the desired recombinant vector was produced. These plasmids were stored in glycerol 60% solution and kept under -80 °C ultra-freezing condition for further transformations into endophytic fungi.

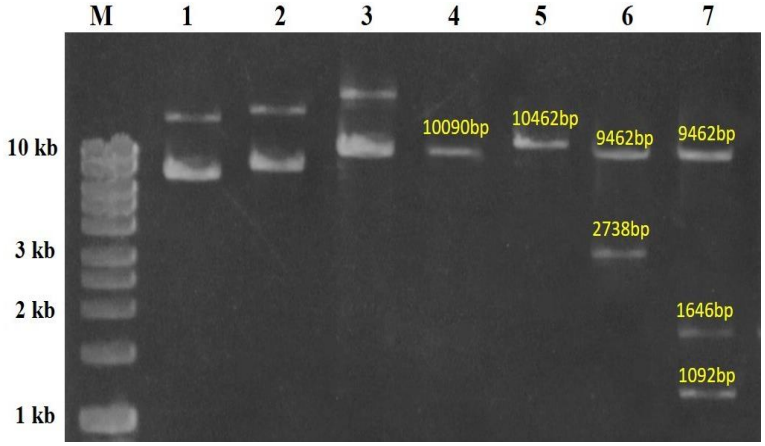

Figure 5. Validating the pGreen3+HYG+PRX1 recombinant plasmids. M: Marker 1kb Bioline, 1: plasmid pGreen3, 2: plasmid pGreen3+PRX1, 3: plasmid pGreen3+HYG+PRX1, 4: pGreen3 cut by *Bam*HI, 5: pGreen3+PRX1 cut by *Bam*HI, 6: pGreen3+HYG+PRX1 cut by *Bam*HI, 7: pGreen3+HYG+PRX1 cut by *Bam*HI and *Kpn*I

DISCUSSION

Our revised *PRX1* CDS was revealed to be highly appropriate for amplification in *E. coli* DH10B competent cells, with the potential to express in endophytic fungi. Several studies indicated the preference for indigenous codons in organisms during transcription and translation (Tokuoka et al., 2008). Therefore, the premature polyadenylation process of mRNA pauses at rare codons leading to interrupt translation elongation, mistakes at protein folding, etc. might occur in bacteria, yeast, plants, mammalian cells, and filamentous fungi that be inserted an exogenic gene (Plotkin & Kudla, 2011). Codon optimization has been demonstrated to be able to alleviate the translation inefficiency caused by ribosomal disruption, significantly stabilize heterologous mRNA transcription, and increase the mRNA levels in some fungal species (Tanaka et al., 2012, 2014; Tokuoka et al., 2008).

The *C. roseus* endophytic fungi have the ability to enhance vindoline content when being inoculated in endophyte-free plants (Pandey et al., 2016). Normally, vindoline production in plants is nonabundant, expensive, and requires a long time. To make these vincristine and vinblastine drugs available at a more affordable cost, producing them by fermentation of the *C. roseus* endophytic fungi seems to be a possible alternative. However, their bioactivity is limited or unstable in practice. Their desired metabolites may reduce significantly during repeated subculturing under monoculture conditions (Kusari et al., 2012). In this case, transforming the endophytes into plants and in planta production can be a more suitable solution than *in vitro* production. Therefore, using a recombinant strategy and optimized *PRX1* genes could improve the stability, efficiency, and bioactivity of the vincristine and vinblastine in the downstream stages.

CONCLUSION

Through cloning steps, we successfully constructed a recombinant plasmid containing the optimized *PRX1* gene. Position from LB to RB of the plasmid includes the *HYG* and *PRX1* gene regions, both genes located between the gpdA promoter and TrpC terminator. The hygromycin resistance ability

enabled this plasmid to be amplified and expressed in endophytic fungi. The recombination plasmid could contribute to the production of the expensive but valuable compounds vincristine and vinblastine in alternative hosts such as endophyte-free plants or endophytic fungi.

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