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Cloning and Functional Validation of Mung Bean *VrPR* Gene

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ABSTRACT

For the purpose of functional validation, the mung bean (*Vigna radiata*) *VrPR* gene was cloned and overexpressed in *Arabidopsis thaliana*. The findings revealed that the ORF of *VrPR* contained 1200 bp, in which 399 amino acids were encoded. Bioinformatics analysis showed that the *VrPR* protein belonged to the NADB Rossmann superfamily, which was one of the non-transmembrane hydrophilic proteins. *VrPR* was assumed to have 44 amino acid phosphorylation sites and be contained in chloroplasts. The *VrPR* secondary structure comprised of random coil, α helix, β angle, and extended chain, all of which were quite compatible with the anticipated tertiary structure. Moreover, analysis of the phylogenetic tree indicated that the soybean *PR* (Glyma.12G222200) and *VrPR* were closely related. Furthermore, chlorophyll content in leaves is markedly increased in Arabidopsis when *VrPR* is overexpressed. Our findings will serve as a reference for more functional studies on the *PR* genes in mung bean.

KEYWORDS

Mung bean; gene cloning; *VrPR*; transgenic arabidopsis; functional verification

1 Introduction

Mung bean (*Vigna radiata*) is an important grain legume that is largely grown in South and East Asia, as a summer species with a short growth period (55–70 days) [1]. Mung bean contains cheap source of carbohydrates (60%–75%), high-quality proteins (20%–27%), folate, and iron [2]. Till now, many people still suffer from hunger and malnutrition, with about 800 million people suffering from hunger in 2021, and the number having risen to 150 million since the outbreak of COVID-19 [3]. Thus, improving the growth and production of mung bean will help solve the food security problem.

In order to enhance the yield of mung beans, a number of studies on the response of associated genes to the regulation of plant growth or the tolerance of environmental stresses have been examined, such as the validation of genes related to salt resistance [4,5], herbicide resistance [6], flowering [7–9], drought resistance [10], cold tolerance in mung bean [11]. Moreover, further researches regarding functional genes mining in mung bean [12,13], genome-wide association analysis [14,15], bioinformatics analysis of key gene families [2,16–18], proteins expression response to diseases [19], somatic cell induction culture [20], seedling germination toxicity [21], and transcriptome analysis in mung bean [22–30] have also been

conducted. Furthermore, the whole genome of mung bean has been sequenced in 2014, which has the length of 543 MB [31]. But studies related to gene functions in mung bean are largely unknown.

In this study, we examined the prochlorophylliate reductase gene *VrPR*, which was associated with the pathway of porphyrin and chlorophyll metabolism production based on the results of the transcriptome sequencing on mung bean leaves [27]. The first enzyme in the *de novo* synthesis pathway of NAD is L-aspartate oxidase, which has been reported to be encoded by the NADB gene, which belongs to the *VrPR* family [32–38]. All living organisms require NAD, a vital cofactor and essential molecule [39,40]. And NADB is mandatory for normal NAD biosynthesis [41–43]. The current study used bioinformatics analysis to clone and characterize the *VrPR* gene. Additionally, *VrPR* overexpression in Arabidopsis was used to study its functional validation.

2 Materials and Methods

2.1 Test Materials

In the present study, the *VrPR* gene was properly cloned using the mung bean genotype Lvfeng no. 2. The overexpressed *VrPR* gene was functionally analyzed using the *Arabidopsis thaliana* Columbia-0 (Col) strain.

2.2 Experimental Methods

2.2.1 Total RNA Extraction and PCR Amplification of VrPR

For the purpose of extracting total RNA, mung bean leaves were collected and rapidly frozen in liquid nitrogen. The TaKaRa RNA extraction kit was used to extract the total RNA following the protocol. The complementary DNA (cDNA) was consolidated through the TaKaRa reverse transcription kit. We designed the primers for the gene of *VrPR*, in which we labeled *VrPR-F* and *VrPR-R* using *HindIII* and *XbaI* enzymatic sites (Table 1). After that, the cDNA of the *VrPR* gene was cloned by PCR amplification with the primers. The PCR reactions were performed under the following conditions: 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 1 min.

Table 1: Primer sequences used for *VrPR* gene cloning

Primer name	Sequences (5′–3′)
VrPR-F	ACCAGTCTCTCTCTCAAGCTTATGGCTCTCCAAGCTGCTTC
VrPR-R	GATACGAACGAAAGCTCTAGATTAGGCCAAACCAACGAGCTTC

2.2.2 Construction of VrPR Gene Overexpression Vector

The PCR-amplified *VrPR* fragment was isolated from an agarose gel and combined with an enzyme-extracted PHB empty vector (Fig. 1). They were then ligated using Tiangen Biologicals' EasyGeno DNA recombinant technology, and transformed into *E. coli* DH5α. The positive colonies were chosen for sequencing after 16 h of culture at 37°C. And the accurate plasmids were isolated for further analysis.

2.2.3 Genetic Transformation and Identification of VrPR Gene

In the present study, we transformed the validated *VrPR* plasmid with a biynary expression vector. We mixed the plasmid with 700 mL of antibiotic-free YEP liquid medium and Agrobacterium tumefacien GV3101 receptor state and incubated it at 28°C for 2–3 h in a shaker at 200 rpm. The bacterial solution was centrifuged at 6000 rpm and resuspended in about 100 μL of supernatant on kanamycin-containing YEP plates. Then we incubated the plates upside down at 28°C in an incubator for 2–3 days. We selected one colony at random for colony PCR identification and used *Agrobacterium tumefaciens*, which carried

the PHB-*PR* overexpression vector, to infest inflorescences of wild-type Arabidopsis. After two infestations, we collected the seeds at maturity and screened them using an Arabidopsis plant known as the T1 plant that was resistant to thaumatin in YEP solid medium. We identified the positive plants by extracting DNA from the leaves of resistant Arabidopsis with a DNA extraction kit from Tiangen Biologicals and testing through PCR.

Figure 1: Figure of the structural information of the PHB over-expression vector

2.2.4 Functional Validation of Transgenic Arabidopsis thaliana

We identified the Arabidopsis homolog genes of *VrPR* through Phytozome (https://phytozome-next.jgi.doe.gov/) and checked the similarity ratio, i.e., 35.80% through DNAMAN software (Fig. 2), which indicated that the Arabidopsis homolog genes had limited influence on the *VrPR* gene.

Furthermore, we measured and analyzed phenotypic and physiological indexes, such as lateral root number, root length, root fresh weight, and chlorophyll.
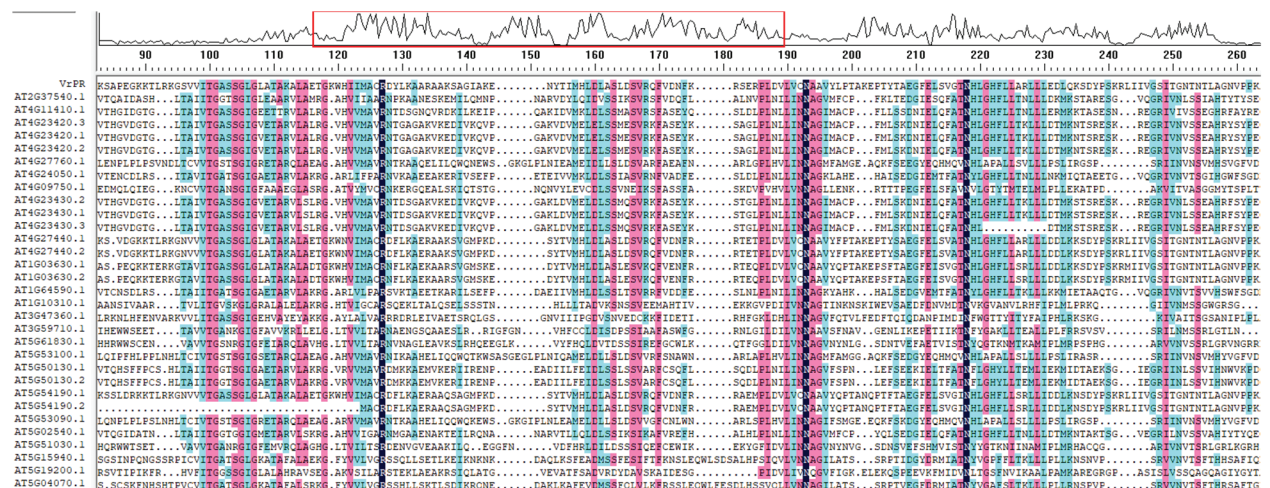

Figure 2: Figure of the sequence similarity alignment between *VrPR* gene and Arabidopsis homolog gene

2.2.5 Bioinformatics Analysis of VrPR Gene

The physicochemical properties of proteins and other traits were identified by the Protparam tool in ExPASy; The conserved domain search tool in the NCBI database is used to find conserved structural domains of gene-encoded proteins; The ProtScale tool in the ExPASy online server is used to predict protein affinity and hydrophobicity; SignalP 5.0 Server is used to predict proteins' transmembrane region; SignalP 5.0 Server is used to predict proteins signal peptide; Softberry Server is used to predict proteins' subcellular region; Softberry Server is used to predict proteins' subcellular region; TMHMM Server 2.0 is used to predict the transmembrane region of proteins; SignalP 5.0 Server is used to predict the signal peptides of proteins; ProtComp 9.0 tool in Softberry Server is used to predict the subcellular localization of proteins; The SOPMA program is used to predict the secondary structure of proteins; The PHYRE2 program is used to predict the protein's tertiary structure; Mega 6.0 software is used to build phylogenetic trees.

2.2.6 Statistical Analysis

We processed and analyzed the experiment data using three different software packages: EXCLE, IBM SPSS Statistics 25, and Origin 2021 Pro. The statistical method is one-way ANOVA, and we express the data in mean and standard deviation (n = 3). The significant differences are significant for p-value < 0.05, very significant for p-value < 0.01, highly significant for p-value < 0.001.

3 Results

3.1 Cloning to Obtain the ORF of VrPR Gene

The information of the *VrPR* gene was retrieved from NCBI with the annotated function asprochlorophyllate reductase (LOC 106764794). By using the CDS sequence of this gene, full-length amplification primers were designed and amplified the gene fragment of about 1200 bp using cDNA from mung bean (Fig. 3). The specific fragment of this gene was ligated to a PHB overexpression vector and verified by colony PCR, which confirmed the 1200 bp gene length (Fig. 4). The results of the bacteriophage sequencing were consistent with the transcriptome sequencing, which indicated the successful cloning of the *VrPR* gene.

3.2 Bioinformatics Analysis of VrPR Protein

3.2.1 Prediction of Conserved Structural Domains of VrPR Protein

Our results showed that the conserved structural domain of VrPR protein was located at the central, non-C-terminal position of the polypeptide chain, which belongs to the NADB Rossmann superfamily of proteins (Fig. 5a). The NCBI blast results confirmed this protein as a protochlorophyllide lipid reductase.

3.2.2 Prediction of Physicochemical Properties of VrPR-Encoded Protein

Physicochemical analyses of this protein indicated its molecular weight 43101.29 Da with molecular formula $C_{1915}H_{3068}N_{530}O_{577}S_{11}$ and the total number of atoms is 6101. This protein contained 399 amino acids, among which 40 amino acid residues (Asp+Glu) have a negative charge and 50 amino acid residues (Arg+Glu) have a positive charge. In addition, it had a 9.23 theoretical isoelectric point (PI) value and a 37.25 instability index, which suggested its stable and hydrophilic nature.

3.2.3 Prediction of VrPR Protein Signal Peptide, Transmembrane Structure and Subcellular Localization

The *VrPR* was found to be a non-secretory and non-transmembrane protein based on signal peptide prediction and transmembrane structure, respectively. Moreover, subcellular localization results showed its location in chloroplasts.

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5'  atggctctccaagctgcttctcttgttcctgcttctttctctcttcctaaagagggaaag   60
1     M   A   L   Q   A   A   S   L   V   P   A   S   F   S   L   P   K   E   G   K
5'  attggtgtgtctctcaaggactccacactgttcggtctttcattgtcagaacctatcaag  120
1     I   G   V   S   L   K   D   S   T   L   F   G   L   S   L   S   E   P   I   K
5'  gctgacttcagctcctctgcattgaggtgcaagagggaatcccagcgaaaaattggtgct  180
1     A   D   F   S   S   S   A   L   R   C   K   R   E   S   Q   R   K   I   G   A
5'  gtgagggcggagacagtggctacagcctctccagcagtcaccaagtctgcaccagaaggc  240
1     V   R   A   E   T   V   A   T   A   S   P   A   V   T   K   S   A   P   E   G
5'  aagaaaacattgaggaaaggtagtgttgtgataactggggcttcctctggattaggcctg  300
1     K   K   T   L   R   K   G   S   V   V   I   T   G   A   S   S   G   L   G   L
5'  gccactgctaaggctttggctgagacaggaaaatggcatataataatggcctgcagggat  360
1     A   T   A   K   A   L   A   E   T   G   K   W   H   I   I   M   A   C   R   D
5'  tacctcaaagctgcaagagctgcaaaatctgctggcatagctaaggaaaattacaccatc  420
1     Y   L   K   A   A   R   A   A   K   S   A   G   I   A   K   E   N   Y   T   I
5'  atgcatttggaccttgcctctcttgacagtgtccgacaatttgttgataacttcaaaaga  480
1     M   H   L   D   L   A   S   L   D   S   V   R   Q   F   V   D   N   F   K   R
5'  tcagaaaggccactagatgtgttggtttgcaatgctgcagtttacttgccaactgcaaag  540
1     S   E   R   P   L   D   V   L   V   C   N   A   A   V   Y   L   P   T   A   K
5'  gaacctacatacactgctgaaggctttgaacttagtgttggaacaaaccatctaggacat  600
1     E   P   T   Y   T   A   E   G   F   E   L   S   V   G   T   N   H   L   G   H
5'  ttcctccttgcacgcctgttgcttgaggacttgcaaaaatctgattacccatcaaagcgc  660
1     F   L   L   A   R   L   L   L   E   D   L   Q   K   S   D   Y   P   S   K   R
5'  ttgatcattgttggttcaatcactgggaacaccaacacattggctggtaatgtacctccc  720
1     L   I   I   V   G   S   I   T   G   N   T   N   T   L   A   G   N   V   P   P
5'  aaggcaaaccttggtgacttgaggggacttcagggtggtttgaatgggctgaacagttca  780
1     K   A   N   L   G   D   L   R   G   L   Q   G   G   L   N   G   L   N   S   S
5'  gccatgattgatggtggagactttgatggtgccaaagcatacaaggacagcaaagtctgt  840
1     A   M   I   D   G   G   D   F   D   G   A   K   A   Y   K   D   S   K   V   C
5'  aatatgctcacaatgcaggaattccacagacgataccacgaggaaactggaatcactttc  900
1     N   M   L   T   M   Q   E   F   H   R   R   Y   H   E   E   T   G   I   T   F
5'  tcatcgctctaccctggttgcattgccacaaccggtttgttcagagagcacattcctttg  960
1     S   S   L   Y   P   G   C   I   A   T   T   G   L   F   R   E   H   I   P   L
5'  ttcagaactctattccctccattccagaagtacataaccaaaggctttgtctcagaagat  1020
1     F   R   T   L   F   P   P   F   Q   K   Y   I   T   K   G   F   V   S   E   D
5'  gaagcaggaaagagacttgctcaggttgtgagtgatccaagtcttacaaaatctggtgtt  1080
1     E   A   G   K   R   L   A   Q   V   V   S   D   P   S   L   T   K   S   G   V
5'  tactggagctggaataaaacatctgcttcatttgagaaccagttatcacaggaggcaagt  1140
1     Y   W   S   W   N   K   T   S   A   S   F   E   N   Q   L   S   Q   E   A   S
5'  gatgctgagaaggctcgtagggtgtgtgggatattagtgagaagctcgttggtttggcctaa  1200
1     D   A   E   K   A   R   R   V   W   D   I   S   E   K   L   V   G   L   *
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Figure 3: Figure of *VrPR* gene ORF sequence and its encoding proteins
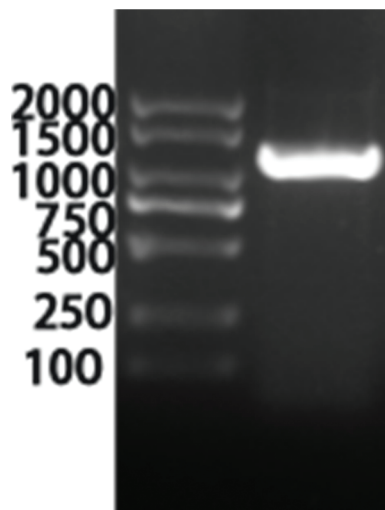
Note: * The terminator.

Figure 4: Figure of the positive figure of PCR cloning, marker is 2000 marker (from top to bottom is 2000, 1500, 1000, 750, 500, 250, 100)

3.2.4 VrPR Protein Phosphorylation Site Prediction

The mung bean VrPR protein had 44 amino acid phosphorylation sites (threshold > 0.5). Among them are 10 phosphorylation sites for threonine, 29 for serine, and 5 for tyrosine, indicating phosphorylation-related activity (Fig. 5b). The phosphorylation of serine sites is predominant in the mung bean VrPR protein.

3.2.5 VrPR Protein Secondary Structure and Tertiary Structure Prediction

The secondary structure of VrPR protein mainly consisted of α-helices (41.60%), irregular coils (35.84%), extended chains (15.04%), and β-turns (7.52%). On the other hand, tertiary structure revealed that VrPR proteins are mainly composed of irregular coils and α-helix. Besides, we performed the soybean gene (*GmPR*) tertiary structure prediction and showed the structural similarity between *VrPR* and *GmPR* (Figs. 5c and 5d). Therefore, the VrPR protein might have the same functions as GmPR.
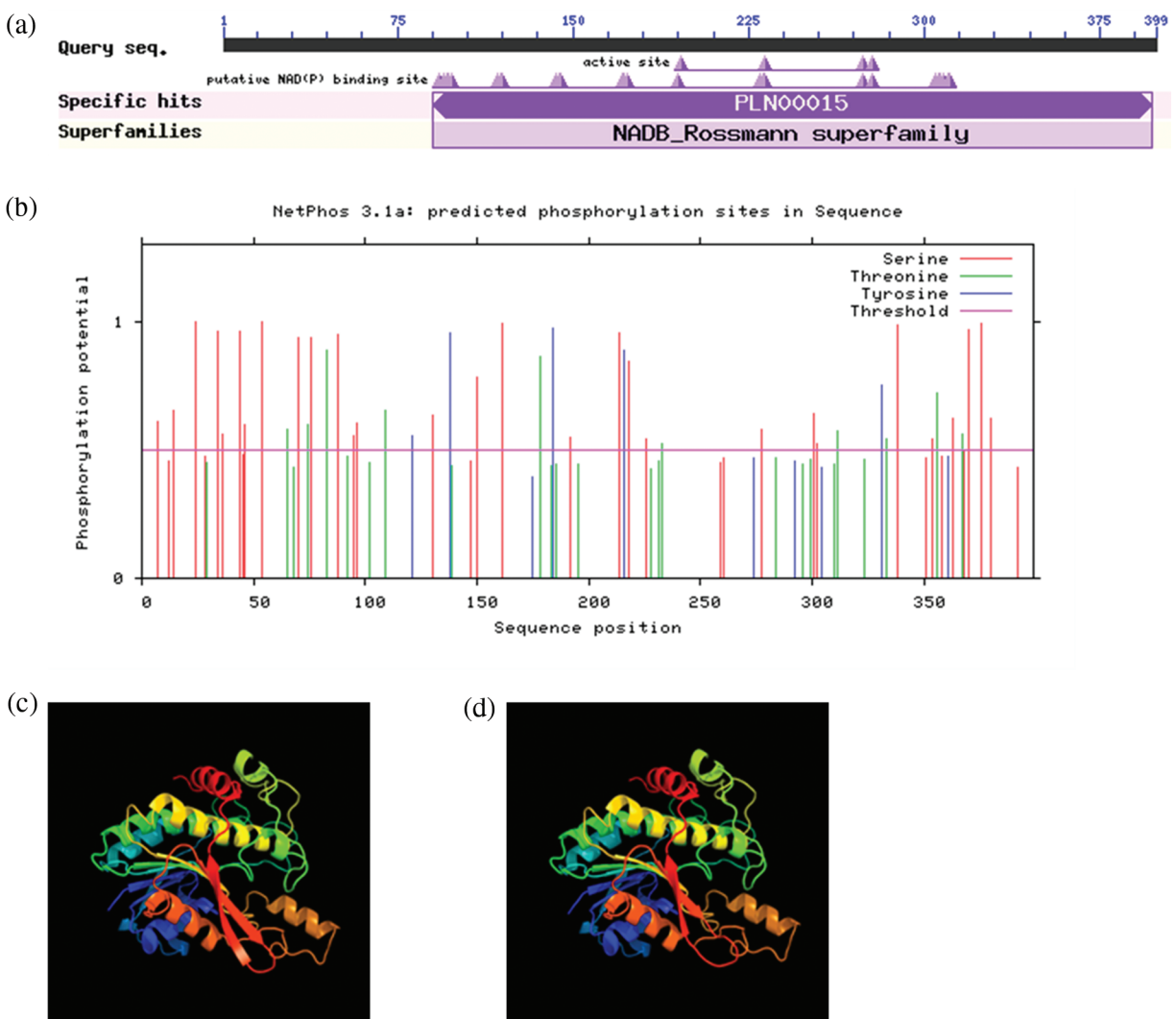

Figure 5: (a) is conservative domain retrieval of VrPR proteins amino acid sequence in NCBI; (b) is the prediction of VrPR protein phosphorylation site; (c) is tertiary protein structure of *VrPR* gene; (d) is tertiary protein structure of *GmPR* gene

3.2.6 VrPR Protein Evolutionary Tree Construction and Multiple Sequence Alignment

The amino acid sequences of *VrPR* were aligned using Phytozome, and phylogenetic analysis of four species sequences showed high concordance. Based on the obtained results, the *VrPR* was found to be closely related to soybean and Arabidopsis proteins and less related to pineapple and rice proteins (Fig. 6). The DNAMAN software results showed that 15 NADB proteins had high homology, which indicated mung bean VrPR proteins as a typical member of the NADB transcription factor family.
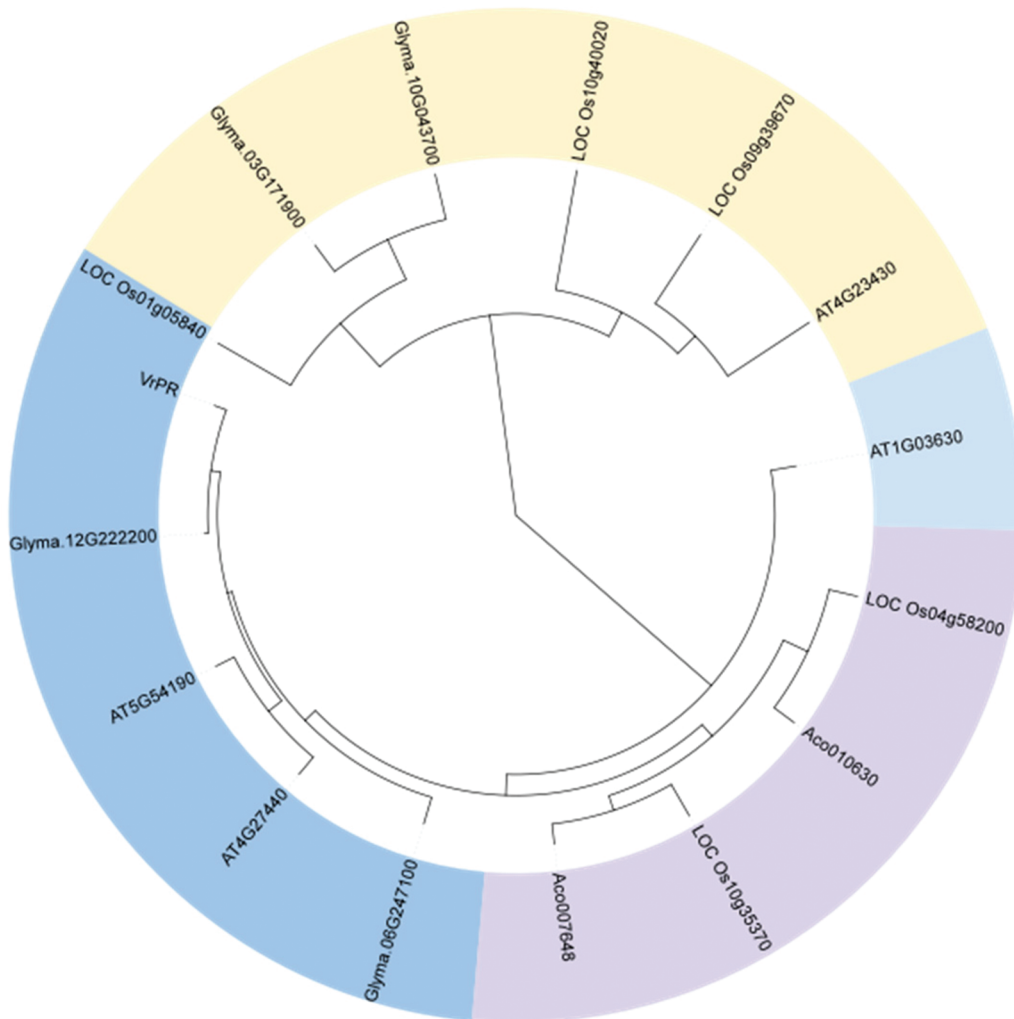
Figure 6: Figure of amino acid sequence alignment of homologous

3.3 Construction of VrPR Gene Overexpression Vector

Cloning of the *VrPR* gene was achieved by overexpressing the PHB vector by adding *Hin*dIII and *Xb*aI enzyme cleavage sites. Later, the targeted gene was ligated to the PHB plasmid with the recombinant enzyme Exnase II. Two pairs of primers, i.e., *PRF* and *PRR*, were used to obtain 1200 bp of fragment length through PCR amplification. Thus, an overexpression vector with the targeted gene was constructed.

3.4 Genetic Transformation and Identification of VrPR Gene

The recombinant plasmid PHB-*PR* was extracted and identified by PCR. Then we purified the plasmid and transferred it to *Agrobacterium tumefaciens* GV3101 receptor cells and selected the positive colonies. The agrobacterium containing the PHB-*PR* plasmid was identified by PCR with two pairs of primers, *PRR* and *PRF*, *HPT-F* and *HPT-R*, and then used to infest Arabidopsis's inflorescence (T0). The infested seeds were collected for resistance screening to obtain Arabidopsis resistant plants (T1). All 16 randomly selected Arabidopsis plants contained the target gene, indicating that the *VrPR* gene had been transferred successfully into Arabidopsis (Fig. 7).
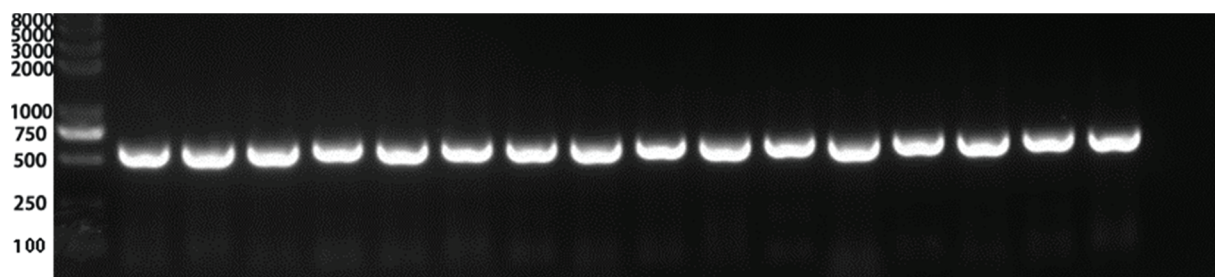

Figure 7: Figure of positive PCR result. Lines 1–15 PCR results (PCR detection of hygromycin resistance gene HPT, 598 bp) showed that 15 lines were positive (Marker: 8000, 5000, 3000, 2000, 1000, 750 (brightest), 500, 250, 100 bp)
Note: Only the first 15 lines were detected. The 15 lines was a positive control. The last lane was a negative control.

3.5 VrPR Transgenic Arabidopsis Root System Phenotype

In Fig. 8, we can see that the *VrPR* transgenic Arabidopsis showed a similar phenotype to the wild type after transformation. On the other hand, no significant differences were found among leaves, root length, and lateral root number, which indicated that genetic transformation did not cause significant phenotypic differences in Arabidopsis.

3.6 Root Fresh Weight and Chlorophyll Index of VrPR Transgenic Arabidopsis

Fig. 9a showed non-significant differences in fresh root weight between transgenic and wild-type plants. These results suggested that genetic transformation had no significant effects on the phenotype of Arabidopsis. Fig. 9b showed extremely significant differences in chlorophyll content between transgenic and wild-type plants. These results suggested that genetic transformation had significant effects on the chlorophyll content of Arabidopsis.

4 Discussion

In this experiment, we selected the gene from the mining of transcriptome analysis and attained a 1200 bp ORF fragment by gene cloning. We analyzed the protein sequence of the gene in bioinformatics and found it is a protochlorophyllide, which belongs to the NADB Rossmann superfamily. And it is predicted to be a hydrophilic protein that is located in chloroplasts in subcellular localization. Serine site Phosphorylation was predominant, and the tertiary protein structure was similar to that of the homologous gene in soybean. The evolutionary tree indicated a closer evolutionary relationship with soybean. By constructing the overexpression vectors and genetically transforming Arabidopsis, we obtained T1 generation seeds and T3 generation pure seeds. We then made a further validation and found that the transformation did not significantly affect the root phenotype. Moreover, it was significantly increased in the transgenic Arabidopsis, and we proposed that overexpression of the *VrPR* gene led to increased chlorophyll in transgenic Arabidopsis.
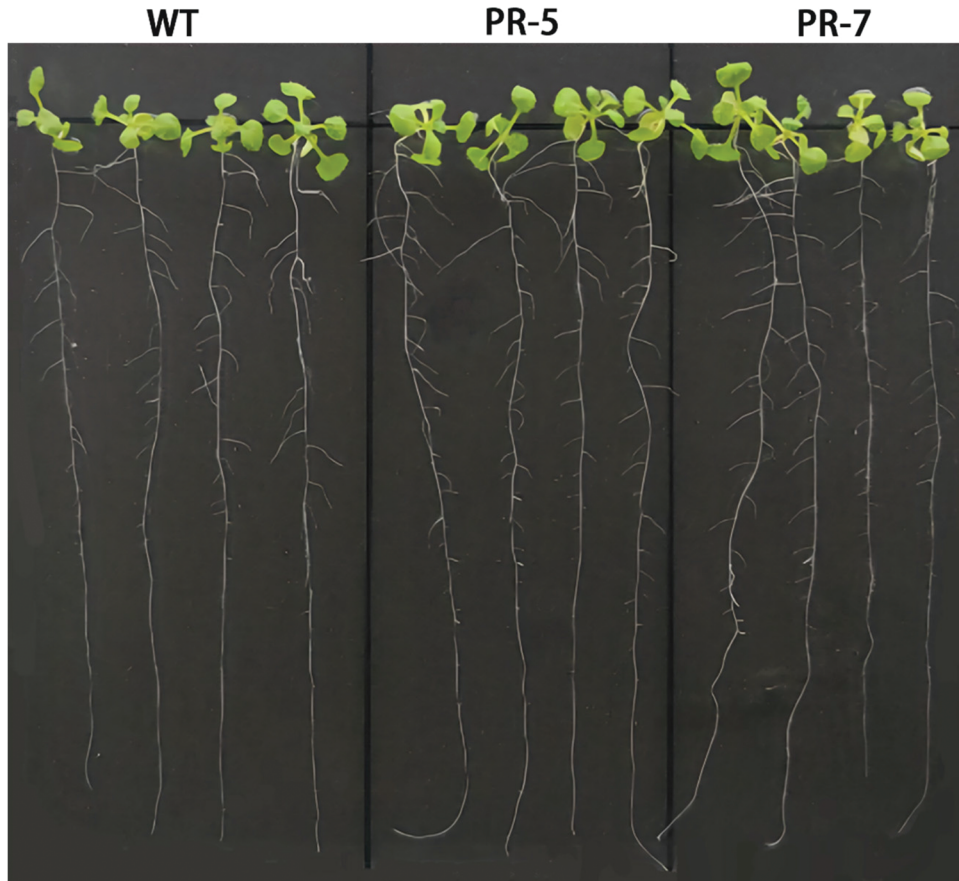
Figure 8: Figure of phenotype picture of *Arabidopsis thaliana*
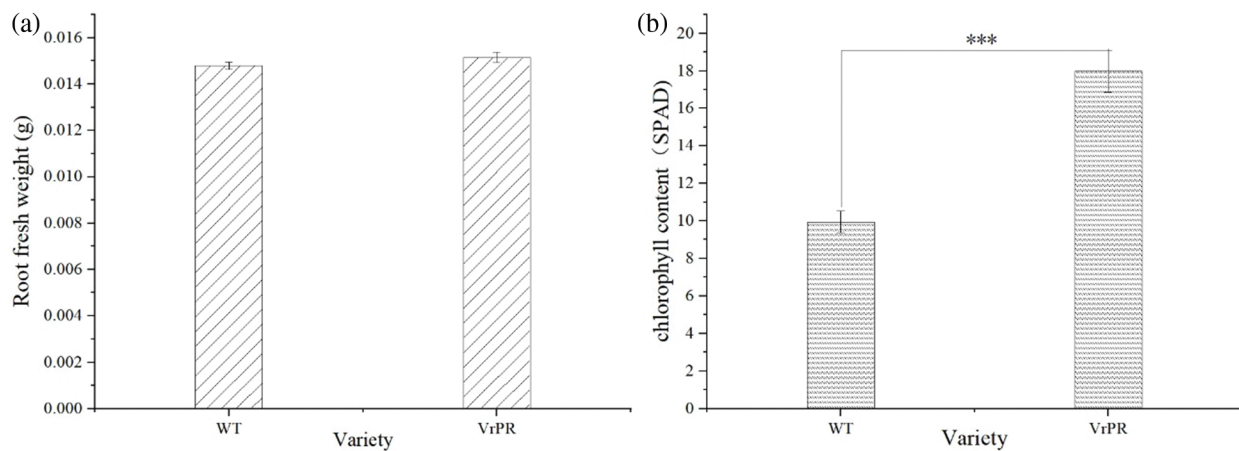

Figure 9: Figure of root fresh weight index of different varieties (a) and figure of chlorophyll index of different varieties (b). The one-way ANOVA was performed between the group and *** indicates that highly significantly; WT indicates wild type of arabidopsis; *VrPR* indicates transgenic arabidopsis of *VrPR*

The *VrPR* gene is blasted as protochlorophyllide with its enzyme activity and gene expression. This enzyme is a key enzyme for chlorophyll synthesis in cyanobacteria, algae, and multicellular plants and is essential for the growth and development of green plants, which can directly affect the greening ability of yellowing seedlings. *VrPR* belongs to the NADB Rossmann superfamily and has binding sites for conserved NADB sequences. NADB can promote the synthesis of NAD coenzymes, indirectly provide energy for chlorophyll synthesis, and improve the viability of Arabidopsis. This may be the way that *VrPR* is involved in increasing the production of chlorophyll.

All of the following studies provided theoretical and data support for the resistance and high yield of mung bean from different directions. For example, research of mining genes related to salt tolerance in mung bean [44], research of genome-wide analysis of different gene families related different stresses in mung bean [2,16,17,45–48], research of validation of mung bean related genes in Arabidopsis [7,49], research of transcriptome analysis of mung bean related different stress [50], and research of reciprocal between different proteins in mung bean [51]. In this experiment, we selected the gene by analyzing the transcriptome [27]. With the bioinformatics analysis of the gene and the functional verification by transgenic Arabidopsis, we found that transgenic Arabidopsis had a significant increase in chlorophyll, and it may be the reason that the overexpression vector promoted Arabidopsis to overexpress the *VrPR* gene and carry out the secretion of related proteins, thus promoting the increase of chlorophyll. However, we can still do gene bioinformatics more comprehensively and deeply. For example, we can still analyze the promoter cis-element and conserved motifs of the *VrPR* gene, etc. The functional validation of Arabidopsis is still in the preliminary stage. For further research, we can focus on experiments such as the interactions of related proteins, the oxidative stress response, and photosynthesis in Arabidopsis.

5 Conclusions

In this experiment, we selected the *VrPR* gene from the analysis of the transcriptome in the preliminary stage of this experiment and performed a functional validation. Including bioinformatics analysis, overexpression vector construction, genetic transformation of Arabidopsis, and functional validation. The results showed that there is a significant increase in chlorophyll in transgenic Arabidopsis, which may be due to the overexpression of the *VrPR* gene, which is a protochlorophyllide that promotes the secretion of chlorophyll in transgenic Arabidopsis. We proposed that overexpression of the *VrPR* gene led to increased chlorophyll in transgenic Arabidopsis, and this can lay the foundation for further experimental research.

Author Contributions: The authors confirm contribution to the paper as follows: Conceptualization, Naijie Feng and Dianfeng Zheng; Data curation, Xiaokui Huang and Yingbin Xue; Formal analysis, Xiaokui Huang and Yingbin Xue; Funding acquisition, Naijie Feng and Dianfeng Zheng; Investigation, Yingbin Xue; Methodology, Yingbin Xue and Dianfeng Zheng; Project administration, Naijie Feng and Dianfeng Zheng; Resources, Hanqiao Hu, Naijie Feng and Dianfeng Zheng; Software, Hanqiao Hu; Supervision, Xiaokui Huang and Yingbin Xue; Validation, Naijie Feng and Dianfeng Zheng; Writing—original draft, Xiaokui Huang; Writing—review & editing, Aaqil Khan and Yingbin Xue. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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