Cloning and Expression Analysis of the Cytokinin Response Regulator Gene PvoRR22 from Plukenetia volubilis (Postprint)

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Abstract

Cytokinin response regulators (RRs) are important components of the cytokinin signal transduction pathway, among which RR22 is a type-C response regulator. Based on the Plukenetia volubilis genome and transcriptome databases, this study cloned the PvoRR22 gene and analyzed its bioinformatics characteristics as well as its expression patterns in different tissues and in inflorescence buds treated with 6-BA. The results showed that: (1) PvoRR22 encodes a protein containing 170 amino acids. The PvoRR22 protein has a molecular weight of 18.65 kDa, a theoretical isoelectric point of 4.54, is a hydrophilic protein, and is localized in the nucleus. (2) Phylogenetic analysis revealed that PvoRR22 from Plukenetia volubilis has a close relationship with RR22 from Ricinus communis and Euphorbia peplus. (3) The promoter sequence of PvoRR22 contains numerous light-responsive, circadian rhythm and stress-responsive elements, as well as hormone-responsive elements for abscisic acid, auxin, and jasmonic acid. (4) PlantRegMap analysis revealed that the expression of PvoRR22 may be regulated by the MYB and ERF transcription factor families. (5) PvoRR22 is mainly expressed in the roots, stems, and shoot tips of Plukenetia volubilis, with the highest expression in roots; in 6-BA-treated inflorescence buds, the expression level of PvoRR22 peaked at 12 h. In summary, it is speculated that PvoRR22 may function in the growth and development of roots, stems, and shoot tips, as well as in cytokinin signal transduction in Plukenetia volubilis.

Full Text

Cloning and Expression Analysis of the Cytokinin Response Regulator Gene *PvoRR22* in *Plukenetia volubilis*

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Abstract: Cytokinin response regulators (RRs) are essential components of cytokinin signal transduction pathways, with RR22 belonging to the type-C RR subfamily. Based on the *Plukenetia volubilis* genome and transcriptome databases, this study cloned the *PvoRR22* gene and analyzed its bioinformatic characteristics and expression patterns in different tissues and in inflorescence buds treated with 6-benzylaminopurine (6-BA). The results revealed that: (1) *PvoRR22* encodes a protein of 170 amino acids with a molecular weight of 18.65 kDa and a theoretical isoelectric point of 4.54, representing a hydrophilic protein localized to the nucleus. (2) Phylogenetic analysis indicated that *PvoRR22* exhibits the closest evolutionary relationship with RR22 homologs from *Ricinus communis* and *Euphorbia peplus*. (3) The *PvoRR22* promoter region contains numerous cis-acting elements responsive to light, circadian rhythms, abiotic stresses, and phytohormones including abscisic acid, auxin, and jasmonic acid. (4) PlantRegMap analysis suggested that *PvoRR22* expression may be regulated by transcription factors from the MYB and ERF families. (5) *PvoRR22* is predominantly expressed in the roots, stems, and stem apices of *P. volubilis*, with the highest expression in roots; in 6-BA-treated inflorescence buds, *PvoRR22* expression peaked at 12 hours post-treatment. These findings suggest that *PvoRR22* may function in the growth and development of roots, stems, and stem apices, as well as in cytokinin signal transduction in *P. volubilis*.

Keywords: *Plukenetia volubilis*, cytokinin response regulators, *PvoRR22*, response elements, expression patterns

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Plukenetia volubilis, commonly known as sacha inchi or Inca peanut, is a perennial woody oilseed vine belonging to the Euphorbiaceae family, native to the tropical rainforests of the Amazon region in South America (Goyal et al., 2022). The seeds are rich in oil and protein with exceptional nutritional value (Torres Sánchez et al., 2023), particularly containing 34% linoleic acid (ω-6) and 51% linolenic acid (ω-3), offering promising applications in food, pharmaceutical, and cosmetic industries (Mhd Rodzi & Lee, 2022; Norhazlindah et al., 2023). Introduced to Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences in 2006, *P. volubilis* has been successfully cultivated in Yunnan, Guizhou, Guangxi, and Hainan provinces of China, as well as in Laos, Thailand, and Myanmar (Pei et al., 2024; Zhang et al., 2024). However, low seed yield has constrained its industrial development. As a monoecious plant with separate male and female flowers, *P. volubilis* produces 50–100 male flowers in the middle and upper portions of the raceme but only 1–2 female flowers at the base (Gillespie, 1993), and this unfavorable female-to-male ratio likely limits seed production. Notably, Fu et al. (2014) demonstrated that exogenous application of the synthetic cytokinin 6-benzylaminopurine (6-BA) can induce the conversion of male floral buds to female floral buds, with some induced female buds developing into normal fruits, thereby increasing seed yield.

Cytokinin is a crucial plant hormone that regulates both plant growth and development (Argyros et al., 2008; Schaller et al., 2014; Joseph et al., 2018; Xu et al., 2020) and mediates stress responses (Cortleven et al., 2019). Cytokinin signal transduction primarily relies on a two-component phosphorelay system (Cheung & Hendrickson, 2010). Cytokinin response regulators (RRs), as key components of this pathway, participate in regulating cell division and differentiation, shoot apical meristem and carpel development, and floral sex determination (Cucinotta et al., 2016; Wang et al., 2017; Rong et al., 2018; Müller et al., 2020; Xue et al., 2020). In *Arabidopsis*, 23 ARR (*Arabidopsis* response regulator) members have been identified and classified into three types (A, B, and C) based on their protein structures (D Agostino et al., 2000). Type-A ARRs possess a conserved Receiver (REC) domain and a short C-terminus (Rashotte et al., 2003). Type-B ARRs contain an additional GARP (Golden2, ARR-B, Psr1) DNA-binding domain that enables them to function as transcription factors (Hosoda et al., 2002; Ishida et al., 2008). Type-C ARRs are structurally similar to type-A but functionally distinct (Kiba et al., 2004).

Current research on type-C RRs has focused primarily on RR22. Studies have shown that *Arabidopsis* ARR22 regulates cytokinin signal transduction through the His-to-Asp phosphorelay pathway, and its overexpression weakens cytokinin signaling, resulting in dwarfism and impaired root development (Kiba et al., 2004). Additionally, ARR22 can act as a suppressor of two-component phosphorelay systems, inhibiting the activation of type-B ARRs (Wallmeroth et al., 2019). Kang et al. (2012) found that ARR22 responds to drought stress, partially dependent on cytokinin receptors AHK2 and AHK3. Akagi et al. (2018) identified a type-C RR22 homolog, *SyGI*, in kiwifruit that resides in a Y chromosome-specific region, suppresses female flower formation, and functions as a male sex-determining gene. Liao (2023) reported that *VfRR22* from tung tree plays a role in early male and female flower development, with expression significantly upregulated at 3 h and 12 h after exogenous 6-BA treatment, peaking at 12 h. Overexpression of *VfRR22* in *Arabidopsis* and tobacco caused abnormal flower development and reduced seed numbers upon 6-BA treatment. Given the important biological functions and functional diversity of RR22 across species, the role of RR22 in *P. volubilis* growth and development warrants further investigation. This study retrieved the ARR22 homolog from *P. volubilis* genome and transcriptome databases, designated as *PvoRR22*, and conducted gene cloning, bioinformatic analysis, subcellular localization, promoter sequence analysis, prediction of upstream transcription factors, and expression analysis in different tissues and 6-BA-treated inflorescence buds to preliminarily understand its potential roles in regulating *P. volubilis* development and its response to cytokinin treatment, thereby laying a foundation for future functional studies.

1.1 Plant Materials and Treatments

Plukenetia volubilis plants cultivated at the *P. volubilis* plantation in Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Mengla County, Yunnan Province, served as experimental material. Root, stem, stem apex, leaf, inflorescence, and young fruit tissues were collected. Following the method of Fu et al. (2014) for 6-BA treatment of inflorescence buds, 20 mg · L^{-1} 6-BA was applied to inflorescence buds approximately 0.5 cm in length on young *P. volubilis* shoots. Inflorescence bud samples were collected at 0, 2, 4, 8, 12, 24, 36, and 48 hours post-treatment, with three biological replicates per sample, immediately frozen in liquid nitrogen, and stored at -80°C. Tobacco (*Nicotiana benthamiana*) used for subcellular localization experiments was the diploid wild type, with seeds maintained in our laboratory.

1.2 RNA Extraction and cDNA Synthesis

Total RNA was extracted from *P. volubilis* samples using the EASYspin Plus Polysaccharide/Polyphenol Complex Plant RNA Rapid Extraction Kit (Aidlab Biotechnologies, Beijing) according to the manufacturer' s instructions. RNA quality and integrity were assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, China) and 1.2% agarose gel electrophoresis, and the extracted RNA was stored at -80°C. Complementary DNA (cDNA) was synthesized from RNA using the PrimeScript™ RT Master Mix reverse transcription kit (Takara Bio, Beijing) following the manufacturer' s protocol.

1.3 Cloning of *PvoRR22* from *P. volubilis*

Based on the retrieved *PvoRR22* sequence, specific primers for amplifying the coding sequence (CDS) and for quantitative PCR were designed using Primer Premier 5 software (Table 1). The CDS of *PvoRR22* (excluding the stop codon) was amplified using Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech, Nanjing). The amplification product was detected by 1.2% agarose gel electrophoresis, gel-purified, and cloned into the pOCA30-GFP vector via homologous recombination. The construct was transformed into *Escherichia coli* DH5α competent cells, and positive clones were selected for plasmid extraction and sequencing by Sangon Biotech (Shanghai).

1.4 Bioinformatic Analysis of PvoRR22

Physicochemical properties of PvoRR22 were predicted using ExPASy-ProtParam (http://web.expasy.org/protparam/). Hydrophobicity was analyzed using ProtScale (http://web.expasy.org/protscale/), and secondary structure was predicted using SOPMA (https://npsa.lyon.inserm.fr). Homologous protein sequences from 16 plant species were obtained from NCBI BLAST (https://blast.ncbi.nlm.nih.gov/), aligned using DNAMAN software, and a phylogenetic tree was constructed using the neighbor-joining (N-J) method in MEGA 7.0.

1.5 Subcellular Localization of PvoRR22

Subcellular localization was predicted using Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). The constructed 35S::*PvoRR22*-GFP plasmid was transformed into *Agrobacterium tumefaciens* GV3101 competent cells. Positive colonies were identified by PCR, cultured to an OD_{600} of 1.0, harvested, and resuspended in 10 mL tobacco infiltration buffer [10 mmol · L^{-1} MES (pH 5.7), 10 mmol · L^{-1} $MgCl_2$]. Acetosyringone was added to a final concentration of 150 µmol · L^{-1}, mixed thoroughly, and incubated at room temperature for 30 min. The bacterial suspension was infiltrated into tobacco leaves using a syringe, and the leaves were cultured in a dark, humid environment for 48 h. After DAPI staining, nuclei and green fluorescence were observed using confocal laser scanning microscopy.

1.6 Promoter Sequence Analysis and Upstream Transcription Factor Prediction of *PvoRR22*

A 1,500 bp promoter sequence upstream of the *PvoRR22* start codon (ATG) was extracted from the local *P. volubilis* genome database (unpublished data) and analyzed using the PlantCARE online tool. PlantRegMap was employed to predict transcription factors potentially binding to the *PvoRR22* promoter.

1.7 Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR was performed using cDNA from different *P. volubilis* tissues and 6-BA-treated inflorescence buds to analyze relative *PvoRR22* expression levels, with *PvoGAPDH* as the internal reference gene and three technical replicates per sample. Reactions were conducted on a Roche LightCycler 480 with a 20 µL reaction mixture containing 10 µL of 2×NovoStart® SYBR qPCR SuperMix Plus, 7 µL of RNase-free H_2O, 1 µL each of forward and reverse primers, and 1 µL of cDNA. The cycling conditions were: 95°C for 3 min, followed by 40 cycles of 95°C for 3 s, 65°C for 15 s, and 72°C for 15 s. Relative expression levels were calculated using the 2^(-ΔΔCt) method.

Table 1 Primers used in this study

Usage	Primer Name	Primer Sequence (5 -3)
Construction of 35S::*PvoRR22*-GFP	PvoRR22 GFP-F	TTTTCTTCGGAGCTTTTCGCGAGCTCATGACTATGAGTTCAGA
	PvoRR22 GFP-R	TCGCCCTTGCTCACCATGGTTCTAGAATAATAGCTGTTGCTGC
RT-qPCR	PvoRR22-F	ATTTCAGTGTGCTTGTGGTGG
	PvoRR22-R	GCCAAATGAACATCTGCCC
	PvoGAPDH-F	TGGCAAGCATATTCAGGCAGGAG
	PvoGAPDH-R	TTGGCTCATCAGGATTGTAGGTATCAG

2.1 Cloning of *PvoRR22*

Using the *Arabidopsis* ARR22 protein sequence (https://www.arabidopsis.org/) as a reference, we screened the local *P. volubilis* genome database and identified the sequence xingyouteng_{10014312}, designated as *PvoRR22*. PCR amplification using *PvoRR22*-specific primers yielded a specific band of approximately 500 bp [Figure 1: see original paper], consistent with the 513 bp CDS length in the database. The amplified product was gel-purified and ligated into the pOCA30-GFP vector via homologous recombination. Sequencing of positive clones confirmed that the obtained sequence matched the original database sequence.

Figure 1 PCR-amplified product of *PvoRR22* CDS. M: Marker 5,000; 1: PCR-amplified product.

2.2 Bioinformatic Analysis of PvoRR22

The *PvoRR22* CDS comprises 513 bp, encoding a 170-amino-acid protein with a molecular weight of 18.65 kDa and a theoretical isoelectric point of 4.54. The protein contains 26 negatively charged and 13 positively charged amino acids, with an instability index of 39.46, indicating relatively stable protein structure. Hydrophobicity analysis revealed maximum hydrophilicity (1.89) at cysteine residue 93 and minimum hydrophilicity (-2.80) at aspartic acid residue 11 and lysine residue 14, with an average hydrophilicity of -0.24, classifying PvoRR22 as a hydrophilic protein [Figure 2A: see original paper]. Transmembrane domain analysis confirmed the absence of transmembrane regions, indicating PvoRR22 is not a membrane protein [Figure 2B: see original paper]. Secondary structure prediction showed that PvoRR22 consists of 44.71% α-helices, 44.21% random coils, and 11.18% β-turns [Figure 2C: see original paper]. Conserved domain analysis using the NCBI Conserved Domain Database identified a REC domain in the protein sequence [Figure 2D: see original paper], confirming that PvoRR22 belongs to the RR gene family.

Figure 2 Bioinformatic analysis of PvoRR22. A: Hydrophilicity prediction; B: Transmembrane domain prediction; C: Secondary structure prediction; D: Conserved domain prediction.

RR22 protein sequences from 16 plant species including *Arabidopsis*, grape, and castor bean were downloaded from NCBI, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 7.0. The results showed that *PvoRR22* is most closely related to RR22 from castor bean (*Ricinus communis*), followed by *Euphorbia peplus*, both belonging to the Euphorbiaceae family, while showing more distant relationships with RR22 from other species [Figure 3: see original paper].

Figure 3 Phylogenetic tree of PvoRR22 and homologous proteins from other species.

2.3 Promoter Analysis of *PvoRR22*

PlantCARE analysis revealed that the *PvoRR22* promoter region contains eight types of light-responsive elements, including LAMP-element, G-box, GATA-motif, Sp1, Box 4, GT1-motif, GA-motif, and Gap-box , suggesting that *PvoRR22* likely participates in light signaling pathways to regulate *P. volubilis* development. Additionally, circadian and stress-responsive elements were identified, indicating potential roles in circadian regulation and responses to abiotic stresses such as drought and low temperature. Hormone-responsive elements were also detected, including an abscisic acid-responsive element (ABRE), an auxin-responsive element (TGA-element), and a jasmonic acid-responsive element (TGACG-motif), suggesting that *PvoRR22* expression may be regulated by multiple phytohormones.

Table 2 Cis-acting elements and their functions in the *PvoRR22* promoter region

Cis-acting Element	Function
Circadian	Circadian control element
MBS	Drought-responsive element (MYB binding site)
TGA-element	Auxin-responsive element
TGACG-motif	MeJA-responsive element
CGTCA-motif	MeJA-responsive element
LTR	Low-temperature responsive element
MBS	Flavonoid biosynthesis responsive element (MYB binding site)
ABRE	Abscisic acid responsive element
LAMP-element	Light responsive element
G-box	Light responsive element
GATA-motif	Light responsive element
Box 4	Light responsive element
GT1-motif	Light responsive element
GA-motif	Light responsive element
Gap-box	Light responsive element

2.4 Prediction of Transcription Factors Binding to *PvoRR22*

PlantRegMap analysis of the *PvoRR22* promoter predicted 176 transcription factors that may bind to this region. The MYB and ERF families were most abundant, each with over 30 members, followed by MIKC-MADS (26 members) and G2-like (21 members) families. Additionally, HD-ZIP, GATA, and NAC families were represented by 11–14 members each, while other families had fewer (1–4) members [Figure 4: see original paper]. These results suggest that *PvoRR22* expression is primarily regulated by MYB and ERF transcription factor families.

Figure 4 Analysis of transcription factors binding to the *PvoRR22* promoter.

2.5 Subcellular Localization of PvoRR22

Tobacco leaves infiltrated with *Agrobacterium* GV3101 carrying the 35S::*PvoRR22*-GFP plasmid were cultured in darkness for 48 h and observed using confocal laser scanning microscopy. The green fluorescence of GFP-tagged PvoRR22 protein colocalized with DAPI-stained blue nuclei, demonstrating that PvoRR22 localizes to the nucleus [Figure 5: see original paper].

Figure 5 Subcellular localization of PvoRR22.

2.6 Expression Analysis of *PvoRR22* in Different *P. volubilis* Tissues

RT-qPCR analysis of *PvoRR22* expression in six *P. volubilis* tissues (root, stem, stem apex, leaf, inflorescence, and young fruit) [Figure 6A: see original paper] revealed significant differential expression. *PvoRR22* showed the highest expression in roots, relatively high expression in stems and stem apices, and lower expression in leaves, inflorescences, and young fruits [Figure 6B: see original paper].

Figure 6 Expression analysis of *PvoRR22* in different *P. volubilis* tissues. A: Six tissues used for expression profiling; B: Relative expression levels of *PvoRR22* in different tissues. *** indicates extremely significant differences compared to other tissues ($P < 0.001$).

2.7 Expression Analysis of *PvoRR22* in 6-BA-Treated Inflorescence Buds

As critical components of cytokinin signal transduction, RRs participate in numerous cytokinin-regulated developmental processes. To investigate the cytokinin responsiveness of *PvoRR22*, RT-qPCR was performed to analyze its relative expression in inflorescence buds [Figure 7A: see original paper] at various time points after 6-BA treatment. The results showed that *PvoRR22* expression was significantly upregulated at 12 h post-treatment, reaching its peak, before returning to normal levels [Figure 7B: see original paper].

Figure 7 Expression analysis of *PvoRR22* in 6-BA-treated inflorescence buds. A: Inflorescence buds used for 6-BA treatment; B: Relative expression levels of *PvoRR22* at different time points after 6-BA treatment. *** indicates extremely significant differences compared to the control ($P < 0.001$).

Discussion and Conclusion

The biological functions of RR gene family members have been extensively reported in multiple species including the model plant *Arabidopsis*, particularly regarding RR22' s role in plant development and floral sex determination (Akagi et al., 2018; Liao, 2023). However, the function of RR22 in *P. volubilis* remains uncharacterized. To explore its potential roles in *P. volubilis* development and

cytokinin signal transduction, this study identified *PvoRR22* from the *P. volubilis* genome, conducted comprehensive bioinformatic and expression analyses, and preliminarily elucidated its possible involvement in cytokinin-regulated inflorescence bud development.

The PvoRR22 amino acid sequence contains the characteristic REC domain of the RR family but lacks the DNA-binding GARP domain, confirming its classification as a type-C RR (Kiba et al., 2004; Ishida et al., 2008). Phylogenetic analysis revealed the highest homology with RR22 from castor bean and *Euphorbia peplus*, both Euphorbiaceae members, while showing distant relationships with cacao and durian homologs. This suggests that RR22 protein structure is evolutionarily conserved, though interspecific homology differences may reflect functional diversification (Pils & Heyl, 2009). In eukaryotes, transcriptional regulation primarily occurs through interactions between cis-acting elements and trans-acting factors (Cui et al., 2023). Promoter analysis of *PvoRR22* identified multiple light-responsive, stress-responsive, and hormone-responsive elements (auxin, jasmonic acid, and abscisic acid). Previous studies showed that type-A ARR4 interacts with phytochrome B to regulate red light signaling (Sweere et al., 2001), suggesting PvoRR22 may also participate in photosynthesis or light signaling regulation in *P. volubilis*. The presence of drought-responsive elements aligns with Kang et al. (2012) findings on ARR22' s drought responsiveness, indicating that PvoRR22 may function in abiotic stress responses. While Geng et al. (2022) identified abscisic acid (ABA) and gibberellin (GA) response elements in *Jatropha curcas* JcRR promoters, the auxin, jasmonic acid, and ABA elements in *PvoRR22* suggest it may be regulated by multiple hormones to influence *P. volubilis* development.

The MYB family, one of the largest plant transcription factor families, extensively participates in hormone signal transduction and biotic/abiotic stress responses (Li et al., 2019). The enrichment of multiple MYB family members among predicted *PvoRR22*-binding transcription factors suggests that PvoRR22 may be regulated by MYBs to execute its functions. Additionally, several ethylene response factor (ERF) family members were enriched in the *PvoRR22* promoter. Since Pan et al. (2021) demonstrated that cucumber *CsERF* genes are involved in female flower development and sex determination, PvoRR22 may similarly be regulated by ERFs to function in *P. volubilis* floral sex development.

Nuclear localization of PvoRR22, demonstrated by colocalization of GFP fluorescence with nuclear staining in tobacco leaves, suggests it functions in the nucleus, consistent with previous reports (Powell & Heyl, 2023). Tissue expression profiling revealed highest *PvoRR22* expression in roots, followed by stem apices and stems, indicating its primary influence on root, stem, and shoot apex development—a pattern similar to tung tree *VfRR22* expression (Liao, 2023). Although *PvoRR22* expression was low in control inflorescence buds, it was significantly upregulated at 12 h after 6-BA treatment, confirming its cytokinin responsiveness. Future studies should examine its expression in 6-BA-treated

roots, stems, and shoot apices, as well as in male and female floral buds at different developmental stages, to further elucidate its biological functions in these tissues.

In summary, *PvoRR22*, as a type-C RR member, appears to be regulated by multiple signals and may function in root, stem, and shoot apex development in *P. volubilis*. Its responsiveness to cytokinin treatment suggests potential involvement in cytokinin-regulated inflorescence bud development. These findings provide a foundation for further functional characterization of *PvoRR22* and warrant investigation into its potential role in regulating floral sex differentiation in *P. volubilis*.

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Note: Figure translations are in progress. See original paper for figures.