Cloning and Developmental Expression Analysis of the CYP71 Gene in Malania oleifera Fruit (Postprint)

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Abstract

Cyanogenic glycosides derived from amino acids are secondary metabolites produced through the catalysis of amino acids by plant cytochrome P450 monooxygenases (CYP), and are associated with plant defense and resistance to abiotic stress. In this study, through analysis of transcriptome data from Malania oleifera, we isolated and cloned a cytochrome P450 gene (designated MoCYP71, GenBank accession number MK172858), conducted bioinformatics analysis, and detected the expression patterns of this gene during different fruit developmental stages. The results revealed that the MoCYP71 gene comprises 1,572 bp, encoding 523 amino acids. The cDNA sequence of this gene exhibits 88% identity with the mRNA sequences of CYP71 genes from Coffea eugenioides (XM_027319282) and Coffea arabica (XM_027213456). The protein has a relative molecular mass of 58,976.54, a theoretical isoelectric point (pI) of 8.10, a molecular formula of C2675H4184N704O744S27, and an instability index (II) of 40.84, classifying it as an unstable protein. The protein lacks a signal peptide, localizes to the secretory pathway, contains two transmembrane structures positioned at amino acids 20-37 and 311-333 as transmembrane hydrophobic helices, and is anchored to organelles. The MoCYP71 protein harbors conserved domains characteristic of the CYP family, including a proline-rich region (PPSPPRLP), K-helix (KETFR), I-helix (GGIDTS), PERF domain (PERF), and heme-binding domain (FGAGRRICPG), and clusters with CYP71E family proteins from Theobroma cacao, Durio zibethinus, and Sorghum bicolor (GenBank accession numbers EOX92908.1, XP_022773875.1, and AAC39318.1, respectively). Following flower withering, the expression level of MoCYP71 gradually decreased (1 month post-withering > 2 months > 3 months), but increased sharply at 4 months post-withering. This study holds significant importance for investigating pest defense mechanisms, tissue maturation processes, and the discovery of bioactive secondary metabolites in Malania oleifera.

Full Text

Cloning of the CYP71 Gene from *Malania oleifera* and Expression Analysis During Different Fruit Developmental Stages

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Abstract

Cyanogenic glucosides derived from amino acids are plant secondary metabolites catalyzed by cytochrome P450 monooxygenases (CYP) and are associated with plant defense and stress resistance. In this study, we analyzed the transcriptome data of *Malania oleifera* and isolated and cloned a cytochrome P450 gene (designated *MoCYP71*, GenBank accession number MK172858). Bioinformatics analysis was performed, and the expression pattern of this gene during different fruit developmental stages was examined. The results showed that the *MoCYP71* gene contains 1,572 bp and encodes 523 amino acids. The cDNA sequence of this gene shares 88% identity with the mRNA sequences of *CYP71* genes from *Coffea eugenioides* (XM_027319282) and *Coffea arabica* (XM_027213456). The relative molecular mass of the protein is 58,976.54, with a theoretical isoelectric point (pI) of 8.10 and a molecular formula of C H N O S . The instability index (II) is 40.84, indicating that it is an unstable protein. The protein lacks a signal peptide, is located in the secretory pathway, and contains two transmembrane structures at amino acid positions 20–37 and 311–333, which are transmembrane hydrophobic helices that anchor the protein to organelle membranes. The MoCYP71 protein contains conserved domains characteristic of the CYP family, including a proline-rich region (PPSPPRLP), K-helix (KETFR), I-helix (GGIDTS), PERF domain (PERF), and the heme-binding domain (FGAGRRICPG). Phylogenetic analysis clustered MoCYP71 with CYP71E family proteins from *Theobroma cacao*, *Durio zibethinus*, and *Sorghum bicolor* (GenBank accessions EOX92908.1, XP_022773875.1, and AAC39318.1, respectively). Expression analysis revealed that *MoCYP71* transcript levels decreased gradually during the first three months after flowering (1 month > 2 months > 3 months), but increased sharply in the fourth month after flowering. This study provides an important foundation for investigating pest defense, tissue maturation, and the discovery of bioactive secondary metabolites in *M. oleifera*.

Keywords: *Malania oleifera*, CYP71 gene, cyanogenic glucosides, bioinformatics analysis, gene expression

Introduction

Malania oleifera, belonging to the family Olacaceae, is a monotypic endangered plant species endemic to China, distributed only in southeastern Yunnan and western Guangxi (Liu et al., 2007). During fruit maturation, the fruits and branches of *M. oleifera* contain small amounts of cyanogenic compounds such as phenylacetonitrile and cyanophenyl benzoic acid derivatives (Tang et al., 2013). This occurs because plants require ethylene synthesis during maturation, which concurrently produces small amounts of cyanogenic glucosides (Gleadow & Moller, 2014). Typically, cyanogenic glucosides exist stably in cellular compartments (Morant et al., 2008), but when plants are attacked by insects, these compounds are degraded into ketones and cyanide to defend against herbivory (Anne Vinther et al., 2008; Jorgensen et al., 2011).

Cyanide is highly toxic to humans; acute cyanide poisoning inhibits cytochrome aa3 oxidase activity in mitochondria, blocks cellular respiration, and can be life-threatening (Nelson, 2006a). The biosynthetic pathway of cyanogenic glucosides was first elucidated in sorghum (*Sorghum bicolor*) (Jones et al., 1999; Gleadow & Moller, 2014) and involves only three structural genes: two membrane-anchored multifunctional cytochrome P450 enzymes (CYP79A1 and CYP71E1) and a soluble UDPG-glucosyltransferase (UGT85B1) (Jones et al., 1999; Bak et al., 1998; Halkier et al., 1995). CYP79A1 catalyzes the conversion of tyrosine to *p*-hydroxyphenylacetaldoxime, while CYP71E1 (also known as 4-hydroxyphenylacetaldehyde oxime monooxygenase) converts *p*-hydroxyphenylacetaldoxime to *p*-hydroxyphenylacetonitrile (Xie et al., 2017). UGT85B1 then adds a glucose moiety to *p*-hydroxymandelonitrile to produce dhurrin. The CYP71E1 enzyme from sorghum belongs to the CYP71 family (Xie et al., 2017). However, the CYP71 family exhibits extensive neofunctionalization and subfunctionalization through gene duplication and recombination. For example, in the dicot *Arabidopsis thaliana*, 52 members of the CYP71 family cover only two subfamilies, CYP71A and CYP71B (Paquette et al., 2000; Nelson et al., 2004), demonstrating remarkable diversity among CYP71 homologs (Jorgensen et al., 2011). This diversity makes functional characterization of individual CYP71 family genes challenging. While CYP71 family genes have been isolated from various plants including *Arabidopsis* (Bak et al., 2001) and soybean (Guttikonda et al., 2010), no studies on CYP71 genes in *M. oleifera* have been reported. In this study, we performed transcriptome sequencing on three-month-old *M. oleifera* fruits, identified a CYP gene (designated *MoCYP71*) from the transcriptome data, cloned its full-length cDNA using RT-PCR, predicted its potential function through bioinformatics analysis, and examined its expression during different fruit developmental stages. This work lays the foundation for further investigation of secondary metabolite biosynthesis in *M. oleifera* fruits and the defense mechanisms against abiotic and pathogen stresses.

Materials and Methods

1.1 Plant Materials

Fruits were collected from *M. oleifera* trees growing in Yanla Village, Jiumo Township, Guangnan County, Wenshan Prefecture, Yunnan Province, at 1, 2, 3, and 4 months after flowering. Leaves were also collected as controls. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until use.

1.2 Transcriptome Analysis and RNA Extraction

Three-month-old *M. oleifera* fruits were sent to BGI for transcriptome sequencing. CYP71 genes in the fruit transcriptome were identified by local BLAST analysis of unigenes and annotation against the NR, NT, Swiss-Prot, KEGG, COG, and GO databases. Total RNA was extracted from *M. oleifera* fruits and leaves using the Qiagen Plant RNA Extraction Kit according to the manufacturer' s instructions. RNA integrity was assessed using 1% agarose gel electrophoresis. One microgram of high-quality RNA was reverse-transcribed to cDNA, which was stored at -20 °C.

1.3 Cloning of the *MoCYP71* Gene

Based on analysis of the *M. oleifera* transcriptome data, a highly expressed CYP gene with a complete open reading frame was identified. Specific primers containing the start codon (MoCYP71F: 5' -ATGCACTTGAGCTTCCAAAG-3') and stop codon (MoCYP71R: 5' -GGTATAATTGGGATGCATAG-3') were designed. Using fruit cDNA as template, PCR amplification was performed with HiFi high-fidelity polymerase. The target fragment was gel-purified, ligated into the pGMT vector, transformed into competent cells, and positive clones were screened by colony PCR. Plasmids from positive clones were sequenced by Shanghai Sangon Biotech. Sequence alignment confirmed the full-length *MoCYP71* gene, which was stored permanently at -80 °C.

1.4 Bioinformatics Analysis of MoCYP71

The *CYP71* gene sequence was translated using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Physicochemical properties, conserved domains, and transmembrane domains of the MoCYP71 protein were analyzed using ProtParam (https://web.expasy.org/protparam/), CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). Amino acid sequences were aligned using DNAMAN to identify conserved domains. The MoCYP71 protein was aligned with known CYP proteins from other plants, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA7 software.

1.5 Expression Analysis of *MoCYP71* in Different Tissues

Two micrograms of RNA from different *M. oleifera* tissues were reverse-transcribed to synthesize the first-strand cDNA. Using tissue-specific cDNA as template, quantitative real-time PCR was performed with specific primers TMoCYP71F (5' -ACCGCCGTCGCCCAATCTTC-3') and TMoCYP71R (5' -AACAGAGCATGAGGACTTGG-3'), with the *elongation factor 1-alpha* gene as an internal reference. The reaction mixture contained 12.5 µL of 2×SYBR Green master mix (with ROX), 0.5 µL each of primers TMoCYP71F and TMoCYP71R, 1 µL of cDNA, and nuclease-free PCR water to a final volume of 25 µL. PCR conditions were: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. Ct values were collected for analysis after the reaction.

Results

2.1 Cloning of the *MoCYP71* Gene

Analysis of the *M. oleifera* transcriptome data identified a plant cytochrome P450-related DNA sequence. Based on this sequence, specific primers MoCYP71F and MoCYP71R were designed, and PCR amplification using fruit cDNA as template yielded a 1,572 bp cDNA fragment [Figure 1: see original paper], designated as the *MoCYP71* gene (GenBank accession MK172858). BLAST analysis of the *MoCYP71* cDNA sequence against NCBI databases revealed 88% identity with mRNA sequences encoding plant cytochrome P450 genes from *Coffea eugenioides* (XM_027319282) and *Coffea arabica* (XM_027213456).

2.2 Bioinformatics Analysis of the *MoCYP71* Gene and Protein

The *MoCYP71* gene encodes 523 amino acids. BLAST analysis of the amino acid sequence showed 58% identity with the cytochrome P450 protein from *Sorghum bicolor* that encodes 4-hydroxyphenylacetaldehyde oxime monooxygenase (Bak et al., 1998) (GenBank accession AAC39318.1), an enzyme belonging to the CYP71 family. Bioinformatics analysis revealed that the MoCYP71 protein has a relative molecular mass of 58,976.54, a theoretical pI of 8.10, a molecular formula of C H N O S , and an instability index (II) of 40.84, classifying it as an unstable protein. The protein lacks a signal peptide, is located in the secretory pathway, and contains two transmembrane structures at amino acid positions 20–37 and 311–333 that anchor it to organelle membranes as transmembrane hydrophobic helices.

Alignment of the MoCYP71 protein with CYP71 family proteins from sorghum, cassava (*Manihot esculenta*), and *Arabidopsis thaliana* (GenBank accessions O48958, Q6XQ14, and Q9LVD2.1, respectively) revealed that MoCYP71 possesses all conserved domains characteristic of plant cytochrome P450 proteins [Figure 2: see original paper], including the proline-rich region

(PPSPPRLP), K-helix (KETFR), I-helix (GGIDTS), PERF domain (PERF), and the heme-binding domain (FGAGRRICPG). To construct a molecular phylogenetic tree, MoCYP71 was aligned with functionally characterized proteins including 4-hydroxyphenylacetaldehyde oxime monooxygenases from *Theobroma cacao*, *Actinidia chinensis* var. *chinensis*, and sorghum (GenBank accessions EOX92908, PSR86236, and AAC39318); phenylaldoxime oxygenases from soybean (*Glycine max*), plum (*Prunus mume*), and avocado (*Persea americana*) (GenBank accessions AF022157, AB920492, and P24465); terpene synthases from chicory (*Cichorium intybus*), sweet wormwood (*Artemisia annua*), Egyptian henbane (*Hyoscyamus muticus*), and tobacco (*Nicotiana tabacum*) (GenBank accessions E1B2Z9, BAM68808, ABS00393, and AAD47832); and indole biosynthetic enzymes from Madagascar periwinkle (*Catharanthus roseus*), *Arabidopsis thaliana*, sunflower (*Helianthus annuus*), and bishop' s weed (*Ammi majus*) (GenBank accessions ADZ48681, O49342, AEI59779, and Q6QNI4). The phylogenetic analysis [Figure 3: see original paper] showed that MoCYP71 clustered with 4-hydroxyphenylacetaldehyde oxime monooxygenase (CYP71E1) proteins from *T. cacao*, *A. chinensis*, and sorghum, which catalyze the conversion of 4-hydroxyphenylacetaldoxime to 4-hydroxymandelonitrile.

2.3 Expression Analysis of *MoCYP71* in Different Fruit Developmental Stages and Leaves

Using cDNA from fruits at different developmental stages and leaves as templates, expression levels of *MoCYP71* were quantified with specific primers TMoCYP71F and TMoCYP71R. The results [Figure 4: see original paper] showed that *MoCYP71* was expressed in all fruit developmental stages and leaves. Compared with leaf expression (L), *MoCYP71* expression was higher in fruits at all developmental stages. After flowering, *MoCYP71* expression decreased gradually during the first three months (1 month (F1) > 2 months (F2) > 3 months (F3)), but increased sharply in the fourth month (F4).

Discussion

The volatile oil of *M. oleifera* contains up to 64.98% mandelonitrile (Huang et al., 2008), which is generated from cyanogenic glucosides by the addition of glucose (Rustler et al., 2007). Cyanogenic glucosides are widespread secondary metabolites in plants whose biosynthesis is catalyzed by cytochrome P450 monooxygenases (CYP) from amino acid precursors (Bak et al., 1998). The CYP79 family catalyzes the conversion of aliphatic or aromatic amino acids to corresponding aldoximes (Gleadow & Moller, 2014), while CYP71 enzymes catalyze the conversion of aldoximes to cyanogenic glucosides (Bak et al., 1998). CYP79 and CYP71E enzymes involved in cyanogenic glucoside biosynthesis have been reported in sorghum (Bak et al., 1998), cassava (Jorgensen et al., 2011), and yew (*Taxus baccata*) (Luck et al., 2017). However, no CYP family genes involved in cyanogenic glucoside biosynthesis have been reported in *M. oleifera*.

In this study, we cloned the CYP71 family gene *MoCYP71* from *M. oleifera*. Bioinformatics analysis revealed that the physicochemical properties, structural domains, recognition sites, cellular localization, and transmembrane structures of the MoCYP71 protein are similar to those of CYP71 proteins from other plants (Sharafeldin et al., 2017; Boutanaev et al., 2015). The CYP71 family can be divided into many clades based on protein sequence identity: 40% identity defines the same family, >55% identity defines the same subfamily, and >97% identity indicates allelic variants (Nelson, 2006b; Liu et al., 2016). MoCYP71 shares 58% identity with the 4-hydroxyphenylacetaldehyde oxime monooxygenase from sorghum (Bak et al., 1998), suggesting that MoCYP71 may function as a 4-hydroxyphenylacetaldehyde oxime monooxygenase (Xie et al., 2017). Phylogenetic analysis divided CYP71 proteins into four functional clades, reflecting the diverse evolutionary divergence of CYP71 family genes in plants (Jorgensen et al., 2011). In this analysis, MoCYP71 clustered with 4-hydroxyphenylacetaldehyde oxime monooxygenases from *T. cacao*, *A. chinensis*, and sorghum, suggesting that MoCYP71 may catalyze the conversion of 4-hydroxyphenylacetaldoxime to 4-hydroxymandelonitrile. The other clades corresponded to phenylaldoxime monooxygenases, terpene monooxygenases, and indole hydroxylases.

Expression analysis showed that *MoCYP71* transcript levels decreased gradually during the first three months after flowering but increased sharply in the fourth month, suggesting a correlation with fruit maturation in *M. oleifera*. In summary, *MoCYP71* participates in cyanogenic glucoside biosynthesis and is associated with fruit maturation, consistent with previous reports that CYP71 genes are involved in tissue maturation and produce small amounts of cyanogenic glucosides during ethylene biosynthesis (Gleadow & Moller, 2014). Similar expression patterns have been observed for the *CYP71E1* gene in sorghum, where CYP gene expression decreases gradually before maturation but increases rapidly during the maturation phase (Jones et al., 1999; Gleadow & Moller, 2014; Bozak et al., 1990). When plant tissues are damaged, cyanogenic glucosides can be hydrolyzed into toxic compounds such as cyanide and nitriles (Irmisch et al., 2014) to protect mature tissues from herbivores, and the expression pattern of *MoCYP71* during fruit development supports this phenomenon. This study identified and cloned the *MoCYP71* gene through transcriptome analysis, performed bioinformatics analysis, and examined its expression during different fruit developmental stages, providing a foundation for understanding defense mechanisms and fruit maturation in *M. oleifera*.

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