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Modular organization analysis of specific naringin/neoeriocitrin related gene expression induced by UVC irradiation in *Drynaria roosii*☆

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ABSTRACT

Drynaria roosii is a traditional medicinal fern, whose rhizome is particularly valued for its effective flavonoid components of naringin/neoeriocitrin. UV irradiation can induce flavonoid accumulation in plants, however the molecular mechanism of UVC-mediated naringin/neoeriocitrin synthesis in *D. roosii* has never been reported. Our HPLC-tandem mass spectrometry (MS/MS) results showed that UVC irradiation increased naringin/neoeriocitrin contents (NNCs) based on irradiation dosage in *D. roosii*. According to the NGS data, DEG-based heat map analysis revealed up-regulation of naringin/neoeriocitrin synthetic genes was more obvious in 12h UVC irradiation (UV dose = 81.648 kJ m^{-2}) when compared with 24h (UV dose = 163.296 kJ m^{-2}), especially for *PAL*, *C3'H* and *HCT* in old rhizomes. Through systems biology method of modular organization analysis, we clustered 15,678 DEGs into 19 modules, and calculated correlation coefficients between modules and samples, as well as modules and NNCs. Four significant naringin-related modules were achieved and displayed high correlations with specific samples. Moreover, weighted gene co-expression network analysis results discovered that *4CL*, *CHS* and *HCT*, *C3H* acted as the hub responsive genes to UVC irradiation involved in naringin and neoeriocitrin synthesis respectively, and presented high co-expression with MYB/bHLH-regulated DEGs. Overall, we demonstrated that UVC irradiation up-regulated the naringin/neoeriocitrin related gene expression in separate new and old rhizomes to enhance NNCs in a dose-dependent manner. Hormetic UVC dose ranges improving NNCs in *D. roosii* were established for the first time. Our work also provided new insights into the study of secondary metabolites in medicinal plants.

1. Introduction

Drynaria roosii (Nakaike), also called *D. fortunei* (Kunze) J. Sm, a large epiphytic fern of the family Polypodiaceae, known as "Gusuibu", has a long history of use in the treatment of bone injuries, inflammation, hyperlipemia arteriosclerosis. Naringin and neoeriocitrin, sharing highly similar chemical structure, are considered as the main effective compounds of 'GuSuiBu', and Pharmacopoeia of the People's Republic

Abbreviations: bHLH, basic helix-loop-helix; C3H, *p*-coumarate 3-hydroxylase; C3'H, *p*-coumarate 3′-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate CoA ligase; CNR, new rhizome; CNRCK, new rhizome treated for 0 h UVC irradiation; CNR12h, new rhizome treated for 12 h UVC irradiation (81.648 kJ m^{-2}); CNR24h, new rhizome treated for 24 h UVC irradiation (163.296 kJ m^{-2}); COR, old rhizome; CORCK, old rhizome treated for 0h UVC irradiation; COR12h, old rhizome treated for 12 h UVC irradiation; COR24h, old rhizome treated for 24 h UVC irradiation; DEG, differentially expressed gene; FDR, false discovery rate; FPKM, Fragments Per Kb per Million reads; F3'H, flavonoid-3'-hydroxylase; GO, Gene Ontology; HCT, hydroxycinnamoyl transferase; KEGG, Kyoto Encyclopedia of Genes and Genomes; MS, mass spectroscopy; NNC, naringin/neoeriocitrin content; NNS, naringin/neoeriocitrin synthesis; NNRG, naringin/neoeriocitrin related gene; NNRGE, naringin/neoeriocitrin related gene expression; PAL, phenylalanine ammonia lyase; RNA-seq, RNA sequencing; NGS, next-generation sequencing; TF, transcription factor; WGCNA, weighted gene co-expression network analysis

☆ The NGS and HiSeq X Ten data were submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under accession numbers SRP108651.

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of China (2015) reports the drug formulation of 'GuSuiBu' with no less than 0.50% naringin (dried drug). Naringin functions in bone maintenance and can increase the expression of Sema3A and the activation of Wnt/β-catenin signalling to prevent disuse osteoporosis induced by denervation (Ma et al., 2016). Neoeriocitrin even shows a better activity than naringin on proliferation and osteogenic differentiation in MC3T3-E1, acting as a new promising candidate drug (Li et al., 2011). In China, 'GuSuiBu' has been used for 'Qianggu Capsules' listed in Category II New Drug for TCM to treat osteoporosis (Xie et al., 2004). Naringin/neoeriocitrin is a kind of flavonoids, which plays the significant role in plant biological processes of environmental stress resistance, such as protection against UV light damage and oxidative stress.

Plants respond to environmental stimuli in various ways including the production of secondary metabolites of flavonoids, which derive from the general phenylpropanoid pathway and are widespread throughout the plant kingdom in response to a variety of abiotic and biotic stressors (Del Vallea et al., 2018). The flavonoid biosynthetic pathway has been well studied by characterizing T-DNA insertion mutants in *Arabidopsis thaliana*, and it branches out from the general phenylpropanoid pathway. 4-coumaroyl-CoA produces naringin chalcone catalyzed by chalcone synthase (*CHS*) and synthesizes naringenin catalyzed by chalcone isomerase (*CHI*). Subsequently activates flavanone 3-hydroxylase (*F3H*), flavonoid 3'-hydroxylase (*F3'H*), and flavonol synthase (*FLS*), leading to the production of the basic flavonols, kaempferol and quercetin (Xie et al., 2012). In *D. roosii*, naringin and neoeriocitrin are suggested to be achieved from 4-coumaroyl CoA and caffeoyl-CoA catalyzed by p-hydroxycinnamoyl CoA quinate shikimate p-hydroxycinnamoyl transferase (*HCT*), *p*-coumarate 3'-hydroxylase (*C3'H*), *p*-coumarate 3-hydroxylase (*C3H*), *CHS*, *CHI*, *F3'H* and UDP-glucosyltransferase (*UGT*), etc. Our previous work has confirmed the expression of naringin/neoeriocitrin related genes in *D. roosii* exhibited a tissue- and time-specific pattern. The accumulation of naringin/neoeriocitrin is indeed time-consuming for over eight years and is quite different in new and old rhizomes (Sun et al., 2018). Considering the balance of biomass and time, three-year-old *D. roosii* plants are reasonable for improving medicinal components of naringin/neoeriocitrin to meet the growing demand.

Previous reports have confirmed that different types of UV light can increase the content of flavonoids and influence the composition of flavonoids varying from lower fern to higher dicotyledon (Wang et al., 2009). The main components of the acclimation response to natural UVB doses are UVB absorbing flavonoids and other phenolics via regulating UVR8 activation of chalcone synthase (Heijde and Ulm, 2012). Genetic evidence has further proven that *Arabidopsis* mutants *tt4* and *tt5* lacking the expression of *CHS* and *CHI* are more sensitive to UVB and UVC irradiation (Li et al., 1993). While Sheng et al. (2018) indicated that the phenolic content and antioxidant activities of grapes after UVC treatment were always higher than UVB treatment, supported by higher expression of several key genes involved in phenylpropanoid, flavonoid and stilbenoid pathways, such as *PAL*, *CHS*, *F3H*, etc. in response to the UVC treatment. Although most harmful solar UVC is effectively eliminated by stratospheric ozone layer, supplemental application of UVC irradiation on fruit and vegetables is widely adopted for postharvest treatment to improve quality throughout storage. Cold storage (4 °C) in combination with UVC exposure of almost 3 min (2.4 kJ m^{-2}) were applied to achieve high stilbenes and flavonoids accumulation in postharvest Redglobe table grape (Crupi et al., 2013). Flavonoid synthesis is regulated mainly at the level of transcription and is responsive to environmental stimuli (Gou et al., 2011). Transcription factors (TFs) of MYB, basic helix-loop-helix (bHLH) and WD40 protein have been confirmed to be involved in the transcriptional regulation of structural genes of flavonoid biosynthetic pathway in response to different light qualities (Hichri et al., 2011). However, the studies on the detailed molecular mechanisms of UVC-mediated naringin/neoeriocitrin synthesis (NNS) in the medicinal fern *D. roosii* are far from clear.

The advancement in high-throughput technologies in recent years, such as microarray and next-generation sequencing (NGS), has resulted in many large data sets cataloging the biological systems at various levels, so that thousands of genes can be analyzed in one shot (Segal et al., 2003). It is well documented that transcriptionally co-expressed genes tend to be functionally related and interact with each other at physiological or molecular level. One method of identifying interacting gene sets is through the construction of gene co-expression networks, which is constructed through the discovery of nonrandom gene-gene expression dependencies measured across multiple transcriptome perturbations (Ficklin and Feltus, 2011). Weighted gene co-expression network analysis (WGCNA) is a powerful tool for co-expression network, by which a Pearson's correlation matrix is calculated, afterwards transformed into an adjacency matrix. The module eigengene (ME) acting as the first principal component can be regarded as representative of the gene expression profile of a given module. Different modules represent specific biological processes, and highly connected hub genes within the module are often regulatory genes. WGCNA has been also widely applied to cluster gene modules and discover hub genes in various plant species such as white spruce, *Populus*, sunflower and rice (Raherison et al., 2015; Gerttula et al., 2015; Moschen et al., 2016; Tan et al., 2017).

The wild *D. roosii* resources have been over-exploited, and need over eight years for artificial cultivation in the greenhouse to generate equivalent NNCs as wild plants. Therefore, the effective way to improve the naringin/neoeriocitrin products in short time is worth of great concern. While UVC irradiation can induce flavonoids in plants, which can be used as the eco-friendly tools to modify naringin/neoeriocitrin products in *D. roosii*. However, the specific effect of UVC irradiation on the NNS and its detailed induction molecular mechanism are largely unknown. Here, we accurately evaluated the NNCs induced by different doses of UVC irradiation in new and old rhizomes via HPLC–MS/MS. To explain the naringin/neoeriocitrin formation mechanism in *D. roosii* respond to UVC irradiation, we carried out the NGS to achieve abundant databases and analyzed expression profiles of naringin/neoeriocitrin related genes (NNRGs) in separate new and old rhizomes respond to different doses of UVC irradiation based on differently expression genes (DEGs). The results confirmed that UVC irradiation induced higher naringin/neoeriocitrin related gene expression (NNRGE) after 12 h UVC irradiation (UV dose = 81.648 kJ m^{-2}) than 24 h (UV dose = 163.296 kJ m^{-2}), especially in old rhizomes. Systems biology methods of modular organization analysis and WGCNA were applied to further study the specific correlations of gene expression pattern and naringin/neoeriocitrin accumulation, as well as identified hub naringin/neoeriocitrin synthetic genes respond to UVC irradiation in *D. roosii*. Overall, we proposed that UVC irradiation could increase the naringin/neoeriocitrin accumulation in *D. roosii* through inducing the specific NNRGE in separate new and old rhizomes, which depended on the UVC irradiation dose.

2. Materials and methods

2.1. Plant materials and treatments

Drynaria roosii was planted in the greenhouse at the Institute of Botany of the Chinese Academy of Sciences. Three-year-old *D. roosii* plants were irradiated using a UVC lamp (254 nm; GL-15; Toshiba) from a distance of 35 cm (1.89 W m^{-2}) for 12 h (UV dose = 81.648 kJ m^{-2}) and 24 h (UV dose = 163.296 kJ m^{-2}). Each UVC treated rhizome sample was collected and divided into two groups of new and old rhizomes. Three biological replicates per experimental treatment and ten plants per replicate were harvested. All of rhizome samples were immediately frozen in liquid nitrogen, afterwards stored at −80 °C until RNA isolation. Total RNA was obtained from each sample using an RNAprep Pure Plant kit (TIANGEN Biotech, Beijing, China). The quality of RNA was detected by an Agilent 2100 bioanalyzer and a NanoDrop