Improving the Anthocyanin Accumulation of Hypocotyls in Radish Sprouts by Hemin-induced NO

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Abstract

**Background:** The biosynthesis of anthocyanin in the hypocotyls of radish (*Raphanus sativus* L.) sprouts was enhanced by hemin in our preliminary experiments, but the underlying mechanism is unclear. Here, we found that NO (nitric oxide) exerted an essential role in Hemin-regulated anthocyanin biosynthesis, which was supported by the following results.

**Results:** Hemin boosted anthocyanin as well as NO content. NO-scavenger cPTIO (carboxy-PTIO) significantly attenuated hemin-induced increase of anthocyanin content, transcripts of anthocyanin synthesis related genes and positive transcription factors, implying that NO played a prominent role during hemin-induced anthocyanin biosynthesis. Hemin specific inhibitor ZnPP (Zinc Protoporphyrin) strongly reduced anthocyanin content, while, NO donor SNP (Sodium Nitroprusside) addition considerably reversed this inhibition and by contrast, resulted in a significant increase in anthocyanin accumulation, closely paralleling the transcripts of structural genes and transcription factors. Moreover, NO content, NR (nitrate reductase) activity and expression level of NOA (nitric oxide associated factor) were up-regulated by Hemin.

**Conclusions:** Those consequences indicated that NO might work downstream in Hemin-heightened anthocyanin accumulation in radish sprouts.

**Background**

Anthocyanins belong to an essentially family of secondary metabolites, flavonoids, stemmed from the phenylpropanoid pathway and are synthesized chiefly in flowers, fruits and leaves. They have a favorable effect on forming pigmentation patterns and providing with tinting hues such as red, orange, purple and blue[1]. Furthermore, anthocyanins play vital roles in the interplay between plants and their environment, like modulating signals physiologically and developmentally, avoiding damage from lethal UV stress, resisting to fast-growing herbivores and pathogens, appealing to vigorous pollinators and seed dispersers[2–4]. Besides the importance of anthocyanins in plant development and growth, they are receiving plenty of attention from the scientific community due to their relevant health-promoting potential, well-known antioxidant activity, and their noticeable character as functional food factors in the precaution of chronic diseases[5].

The biosynthesis pathway of anthocyanins has been well demonstrated in plants. There are two stages divided among these genes which participate in this pathway, early biosynthesis genes (EBGs) such as phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase(*CHI*), flavanone-3-hydroxylase (*F3H*), and late biosynthesis genes (LBGs) including dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase(*ANS*), leucoanthocyanidin dioxygenase (*LDOX*), UDPglucose: flavonoid-3-O-glucosyltransferase(*UF3GT*)[6].

Anthocyanin synthesis is predominantly subjected to transcriptional regulation that is coordinated by a transcription factor compound consisting of MYB, bHLH, WD-repeat(WDR) proteins, and generally being
called MBW complex[7, 8]. The activity of R2R3-MYB factor determines the pattern and spatial location of anthocyanins[9]. PAP1(Production of anthocyanin pigment1) and PAP2(Production of anthocyanin pigment2) are the part of R3R3-MYB family, AtPAP1 and AtPAP2 encode the AtMYB75 transcription factor and AtMYB90 transcription factor linked with anthocyanin biosynthesis, respectively[10–12]. Anthocyanin accumulation is repressed at darkness mainly due to PAP1 and PAP2 protein are affected by Constitutively Photomorphogenic1/Suppressor of PhyA-105 (COP1/SPA) ubiquity in ligase degradation effect. In fact, anthocyanin biosynthesis is enhanced without light. Many studies demonstrated that COP1/SPA protein is out of the nucleus in light and loses function to regulate transcription factors located in the nucleus. However, light not only can heighten the transcription level of PAP1 and PAP2, but also maintain the stability of the PAP1 and PAP2 protein[13].

Hemin is widely used in food industry, being regarded as food additives and natural iron-supplementary nutrition. For one thing, hemin have higher absorption rate than other inorganic iron supplements such as ferrous sulfate and reduced iron, which will don’t form insoluble iron salts with phytate, carbonate and tannic acid to hinder effective absorption and its non-toxic. For another thing, as a pigment additive, hemin can enhance nutrition and it can avoid from carcinogenic effect.[14, 15]. The commercialized hemin is ferriprotoporphyrin IX compounds, which is a crucial degradable product of hemoglobin[16, 17]. In animals and plants, hemin is considered as a substrate or an inducer of heme oxygenase-1(HO-1), which is regarded as a critical enzyme that catalyzes the decomposition of heme[18]. Exogenous application hemin availablely mitigated cadmium stress, salinity adversity and UV-B radiation through up-regulating the activity of HO-1 and the expression of HO-1[19–21]. As biosynthesis of anthocyanins are deemed to have connection with defense responses of plants against abiotic stresses[22], there may be a relationship between hemin and anthocyanin accumulation, which has previously been reported in plants.

Nitric oxide (NO), generally speaking, is a functional gaseous free radical among animals and plants. According to majority of reports, it’s also remarkably regarded as a signaling molecule in all kinds of physiological activities in plants, including responding to biotic or abiotic stresses[23–25], plant growth and development[26] or iron homeostasis[27]. The biosynthesis pathway of NO was firstly discovered in mammals. Nitrate reductase (NR) and nitric oxide synthase (NOS), in plants, are the prime enzymatic source taking part in the generation of NO[28, 29]. Some studies are reported that NO and HO-1, two signal compounds, have been confirmed to simultaneous involve in diverse processes such as drought tolerance, heavy metal toxicity, adventitious root formation and seed sprout[30, 31]. Lately, it was said that appropriate nitric oxide concentration increased red raspberries quality and maintain high level of anthocyanin content during storage[32]. However, the NO involvement in hemin-facilitating anthocyanin remains unclear.

Radish (Raphanus sativus L.) is an edible root vegetable of the Brassicaceae family with high level of vitamins, minerals, glucosinolates, phenolic components and so on[33]. While, compared with mature root, radish sprouts have been the concentration of many recent researches due to the much higher content of nutrition[34]. Many studies have shown that anthocyanin was accumulated in the hypocotyles of radish sprouts with red skin[35, 36]. In this work, our essential aim was to investigate if hemin affect
the anthocyanin accumulation in radish sprouts and verify the role of NO in this process. Results will contribute us to understanding the way that hemin heighten the anthocyanin content, so as to elevate anthocyanin accumulation of the hypocotyls of radish sprouts.

Results

Effects of hemin on anthocyanin accumulation and endogenous NO production in radish sprouts

To affirm the impact of hemin on accumulating anthocyanin in radish sprout hypocotyls, those samples grown under 1, 10, 25 and 50 µM hemin solution were examined (Fig. 1). Findings showed that the expenditure of hemin could have a higher amount of anthocyanins and NO in the hypocotyls of radish sprouts than that of control group. Therefore, it indicated that hemin treatment triggered the accumulation of anthocyanin and generation of endogenous NO. The anthocyanin content could be increased both under 25 and 50 µM hemin addition, with the highest level under 25 µM. All those expounded that 25 µM hemin was the most appropriate treatment group.

Effects of NO on the accumulation of anthocyanin in hypocotyls of radish sprouts

Proving the correlation between NO and anthocyanin in the hypocotyls of radish sprouts is not ignorable. Hence, an exogenous NO donor, Sodium Nitroprusside (SNP), and a specific NO-scavenger, carboxy-PTIO (cPTIO) were chosen in order to observe anthocyanins content of hypocotyls in radish sprouts. There was a similar trend where sprouts handled with SNP treatment, ranging from 10 to 1000 µM, had an obviously higher quantity of anthocyanins, compared with control group (Fig. 2A). It was prominent that anthocyanins content was at the peak with the employment of 200 µM SNP. In contrast, compared with the control group, anthocyanin accumulation in hypocotyl treated by cPTIO at 50 to 1000 µM was significantly decreased. Apparently, cPTIO treatment had the minimum of anthocyanins at 200 µM (Fig. 2B). It means that climbing NO content could strengthen the ascent of anthocyanin in radish sprouts. On the contrary, scavenging NO could be drastically dropped anthocyanin production.

Nitric oxide participates in anthocyanin accumulation induced by hemin

Subsequently, ZnPP, a specific inhibitor of HO-1[37], was appended into the radish sprouts so as to deeply disclose the interplay between hemin and NO on the anthocyanin accumulation in the hypocotyls of radish sprouts. As the figures were shown, respective disposal of hemin and SNP evidently enhanced anthocyanins content. Obviously, co-treatment between hemin and SNP also sharply heightened this effect. The figure of anthocyanins, however, were slightly descended with the exogenous application of ZnPP or cPTIO treatment. Moreover, the anthocyanin of radish sprouts fostered with hemin and cPTIO
were lower than that with hemin treatment alone. Meanwhile, co-treatment of ZnPP and SNP reversed the inhibitory impact of ZnPP. In addition, the anthocyanin of co-treated group with ZnPP and SNP were higher than that with Hemin and cPTIO (Fig. 3).

Expression of anthocyanin correlative-biosynthesis structural and regulatory genes under unlike treatments

It was necessary to undoubtedly ascertain the effects between hemin and NO about synthesizing anthocyanin, we determined the expression levels of anthocyanin biosynthesis-related structural and regulatory genes in the hypocotyls of radish sprouts. As expected, after seedlings were conducted with hemin, SNP and combination of hemin and SNP separately, the majority of structural genes expression were dramatically up-regulated like PAL, DFR, UF3GT and so on. Furthermore, the addition of ZnPP, conversely, suppressed the hemin-induced phenomenon. Besides, expression level of most structural genes treated with ZnPP and cPTIO was smallest than the rest of treatments. And those genes of ZnPP and SNP co-treatment were higher than that of Hemin and cPTIO (Fig. 4).

It has been reported that the transcription factor MYBs, which regulates the expression level of structural genes, is involved in the biosynthesis of anthocyanin[38]. As shown in Fig. 5, there was an analogical pattern where the expression level of PAP1 was risen as well as anthocyanin content and structural genes expression. PAP2, however, did not exhibit prospective mode.

NO content and biosynthesis-connected enzymes activity and gene expression with various treatments

In order to clarify the changes of endogenous NO under different treatments, the NO content, expression of NO biosynthesis-associated genes and enzymes activities were determined. Consequences in Fig. 6A were optically presented that the hypocotyls of NO content mainly centralized in the tissue of stele. As Fig. 6B-C shown, hemin could boost NO production with comparison of control, which was inferior to effective capacity of SNP treatment. Co-process between hemin and SNP, moreover, could markedly stimulate this promoting influence. In addition, treatment with ZnPP or cPTIO could suppress the NO generation. The content of NO in the co-treatment of ZnPP and cPTIO was the lowest. Furthermore, we found that resembling manner with anthocyanins variation, thus we deduced that endogenous NO had close connection with hemin-promoted anthocyanin accumulation.

To forward make sure this internal principle, we defined expression level of interrelated genes and the activities of two key enzymes which is a crucial element in NO biosynthesis pathway, such as nitrate reductase (NR) and nitric oxide synthase (NOS) (Fig. 7A-B). Testimonies were expounded that the variation of NR activity and the expression of NIA (Nitrate reductase[ NADH]) were positively relevant with the level of endogenous NO, By contrast, the pattern of NOS were not overtly regular.

Discussion
We have sought to answer one crucial question here: How hemin induces anthocyanin accumulation. On the basis of our finding, NO have involvement in this process and this is one of few studies to elaborate the effects of hemin on modulation of anthocyanin biosynthesis as most previous reports were concentrated on the response of hemin on abiotic stresses in plants.

In the present study, we stated that the content of anthocyanin and NO were increased gradually from 0 to 50 µM Hemin treatment in radish sprouts (Fig. 1), which demonstrated that hemin could promote the accumulation of anthocyanin and NO content in the hypocotyls of radish sprouts. We guessed that NO might participate in the adjustment of anthocyanin accumulation. To further indicate whether NO could affect the biosynthesis of anthocyanin in radish sprouts, we verified the anthocyanin contents under treatments of exogenous SNP, and cPTIO. It showed that exogenous addition of SNP significantly rose anthocyanin content in the hypocotyls of radish sprouts (Fig. 2A), but the anthocyanin level in hypocotyls of treatment with cPTIO was lower than that of control (Fig. 2B), suggesting that NO could advance anthocyanin accumulation in the radish sprouts.

Some research showed that NO had cross-talk with HO-1 in some processes, for instance, in the HO-1 signaling responsible for stomatal closure in Vicia faba leaves, NO may function as downstream intermediates[39]. What’s more, NO is participated in hemin-induced adventitious rooting course in cucumber seedlings[17]. It prompted us to thoroughly perceive the interaction between hemin and NO on the accumulation of anthocyanin, and the current study manifested that the supplement of hemin and cPTIO decreased the level of anthocyanin content, compared with hemin treatment alone. In addition, more anthocyanin content was accumulated in hypocotyl of radish sprouts treated with Znpp and SNP co-treatment than that handled with Znpp treatment(Fig. 3). These results revealed that NO might operate on the downstream of hemin to promote the production of anthocyanin.

In parallel with the production of anthocyanin, the relative expression levels of the anthocyanin biosynthesis-related structural genes (PAL, DFR, ANS, UF3GT, LDOX) and regulatory genes (PAP1, PAP2) were all remarkably up-regulated by hemin compared to control. The expression level of anthocyanin synthesis structural genes like PAL, DFR, UF3GT, ANS and LDOX and positive transcription factor PAP1, PAP2 in radish sprouts treated with hemin treatment are higher than hemin and cPTIO co-treatment. PAL, CHS, F3H, DFR, UF3GT, LDOX and transcription factor PAP2 were decreased in hypocotyl of radish sprouts treated with Znpp treatment compared to ZnPP and SNP co-treatment(Fig. 4–5). These consequences explained that the biosynthesis of anthocyanin was commanded by structural genes and transcription factors, which was in accordance with the former findings in apple[40], grape[41] and pear[42]. We adopted accurate fluorescent probes DAF-FM-DA to precisely identify NO content in diverse treatment. NO content of hemin treatment was higher than control. However, this promoting effect was suppressed by hemin and cPTIO co-treatment. In addition, hypocotyl of radish sprouts treated with Znpp have less NO content than co-treatment with Znpp and SNP (Fig. 6) and the activity of NO biosynthesis-related enzymes were assayed to ascertain the fluctuant changes of endogenous NO under disparate treatments. Our studies declared that exogenous employment of hemin significantly rose the NR activity and the transcript level of NIA compared to control and then further promoted the production of NO, while
the change of NOS had no obvious pattern. But the NR activity was reduced by hemin and Znpp co-treatment.

**Conclusion**

Taken together, we proposed a preliminary mechanistic model to expound hemin-induced increase of anthocyanin in radish sprouts as shown in Fig. 8. The addition of Hemin could promote the accumulation of NO by increasing the activities of NOA and NR. NO could up-regulate the relative expression of transcription factors (PAP1 and PAP2), thus increasing the level of structural genes which is related to anthocyanin biosynthesis. The expression of transcription factors (PAP1 and PAP2) were up-regulated by NO, which increased the transcript levels of anthocyanin biosynthesis related structural genes. However, several remaining questions should do more studies in the future work. First, as hemin is an inducer of HO-1, whether hemin promotes anthocyanin biosynthesis through triggering the activity of HO-1 or not. Whether or not the product of hemin like biliverdinbiliverdin (BV), free iron (Fe$^{2+}$) and carbon monoxide (CO) are involved in this process remains to be resolved.

**Methods**

**Plant materials and growth conditions**

*R. Sativus* (L.) cv. Yanghua seeds (Purchase from Nanjing Lvling Seed Industry Co., Ltd.) were singled out and steeped in distilled water for 12 hand burgeoned for 24 h at room temperature in darkness. Healthy seedlings were sorted and planted on paved sterile gauzed in the dark with watering every 12 h for 36 h. They, Subsequently, are treated with diverse reagents and transferred to a white illuminating incubator (Ningbo Haishu Safe Instrument Experimental Factory, Zhejiang, China) for 48 h at room temperature, the lightintensity parameter was 50 ± 5 µmol·m$^{-2}$·s$^{-1}$. Those plants, after that, are harvested and utilized to determine corresponding index.

**Anthocyanin Analysis**

Anthocyanin measurement was carried out as the method was described by[43] with some amendments. 0.5 g fresh hypocotyl placed in 10 ml centrifuge tube, in brief, were thoroughly immersed overnight in 1% methanol hydrochloride for 24 h in the dark. Those are centrifuged in 5,000 g for 10 min at 4°C before supernatant were measured by spectrophotometry (UV-5200 spectrophotometer; Shanghai Metash Instruments Co., Ltd, China).

Anthocyanin content(U·g$^{-1}$FW) = (A$_{530}$-A$_{657}$ × 0.25)/Fresh weigh

**Observation and Analysis of hypocotyl cross section of Radish sprout**
Hypocotyls of radish sprouts in the same part were timely transected with a sharply double-edged blade and accurately monitored under a stereomicroscope (Model Stemi 2000-C; Carl Zeiss, Germany). Then they were shoot with a color photography availably (Powershot A620, Canon Photo Film, Japan).

**NO quantification**

0.5 g Radish hypocotyls were averagely homogenized with 4 mL of 40 mM HEPES buffer (pH 7.2), and then centrifuge for 10 min at 8,000 g at 4°C. The supernatant, being tested in an A012 Nitric Oxide (NO) assay kit, was gathered to inspect NO content (Nitrate reductase method) (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Accurate fluorescent probes DAF-FM-DA (3-Amino,4-aminomethy1-2’, 7’difluorescein, diacetate) were applied to further ascertain the NO level in radish sprout hypocotyls by referring to the instruction manual.

**Determination of NR and NOS activities**

The activity of NR was determined according to the method expounded by Sun [44]. To assay NOS activity, the total protein was extracted as clarified by Lin[45]. NOS activity was subsequently inspected according to Gonzalez[11].

**Gene expression (quantitative real-time PCR)**

Total RNA was separated from 100 mg (fresh weight) of robust radish hypocotyls. Those samples were stored into a sterile mortar, followed by the swift grind course with involvement of liquid nitrogen. Trizol reagent, after the powder being obtained was expended and mingled adequately (Invitrogen, Gaithersburg, MD). And the extracted RNA, afterwards, was disposed with DNase I (RNase-free, Transgen®) at 25 °C for 30 min, followed by performance of reverse transcription in view of the manufacturer’s instruction (TransScript® First-Strand cDNA Synthesis SuperMix, Transgen®). The qRT-PCR reactions were manipulated by utilizing a Mastercycler® ep realplex real-time PCR system (ABI7500, MD, USA) with Bestar® SybrGreen qPCR mastermix (DBI, Bioscience Inc., Germany) in a 20 µL reaction volume. The cDNA were augmented by consuming the primers as shown in Table S1.

**Statistical analyses**

Values analysis was operated with usage of the SPSS statistical software installation package (version 11.0). Differences among treatments were analyzed by one-way analysis of variance (ANOVA) integrated with Duncan's multiple range test, with P < 0.05 as the threshold.

**Abbreviations**

NO: nitric oxide; NR: nitrate reductase; NOA: nitric oxide associated factor; EBGs: early biosynthesis genes; PAL: phenylalanine ammonia lyase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H: flavanone-3-hydroxylase; LBGs: late biosynthesis genes; DFR: dihydroflavonol 4-reductase; ANS: anthocyanidin synthase; LDOX: leucoanthocyanidin dioxygenase; UF3GT: UDPglucose flavonoid-3-O-glucosyltransferase; PAP1: production of anthocyanin pigment1; PAP2: production of anthocyanin
pigment2; COP1/SPA: Constitutively Photomorphogenic1/Suppressor of PhyA-105; cPTIO: carboxy-PTIO; ZNPP: ZincProtoporphyrin; SNP: Sodium Nitroprusside; NIA: (Nitrate reductase[NADH]); BV: biliverdinnobiliverdin; CO: carbon monoxide; HO-1: heme oxygenase-1.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analysed during this study are included in this published article [and its supplementary information files].

**Competing interests**

The authors declare that they have no conflict of interest.

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**Authors’ contributions**

NN designed and guided the experiment. ZL and MY modified and submitted manuscript. HC wrote manuscript and analyzed data. JC provided experimental guidance and ideas. All authors read and approved the final manuscript.

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