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**Cyclic ADP-ribose mediates nitric oxide-guanosine 3',5'-cyclic
monophosphate-induced isoflavone accumulation in soybean sprouts
under UV-B radiation**

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Abstract. In this study, in order to investigate the role of cyclic ADP-ribose (cADPR) in nitric oxide-guanosine 3',5'-cyclic monophosphate (NO-cGMP)-induced isoflavone accumulation in soybean sprouts under UV-B radiation, the sprouts were treated with donors and inhibitors of NO and cGMP as well as cADPR inhibitor. Results showed that NO, with cGMP as a second messenger, activated ADP-ribosyl cyclase (ADPRC), leading to cADPR accumulation under UV-B radiation. cADPR inhibitor suppressed UV-B radiation-induced isoflavone synthesis, the activity, gene and protein expression of chalcone synthase (CHS) and isoflavone synthase (IFS), while this inhibition could be reversed by sodium nitroprusside (SNP) and 8-Br-cGMP. This suggested that cADPR induced by the NO-cGMP pathway was involved in isoflavone synthesis by elevating the activity, gene and protein expression of chalcone synthase (CHS) and isoflavone synthase (IFS). Overall, cADPR mediates NO-cGMP-induced isoflavone accumulation in soybean sprouts under UV-B stress.

Keywords: cADPR; NO-cGMP; UV-B; isoflavone; soybean sprouts

Abbreviations

cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; NO, nitric oxide; SNP, sodium nitroprusside; LY-83583, 6-Anilino-5,8-quinolinequinone; GC, guanylyl cyclase; cGMP, guanosine 3',5'-cyclic monophosphate; ADPRC, ADP-ribosyl cyclase; cADPR, cyclic ADP-ribose; CHS, chalcone synthase; IFS, isoflavone synthase; PAL, phenylalanine ammonia lyase; PR-1, pathogenesis-related

1 protein; ABA, abscisic acid; CK, control group.

Introduction

Isoflavones, an important group of flavonoids, belong to a family of polyphenols and are synthesized predominantly in legumes (Devi and Giridhar 2014). Enrichment of isoflavones has been investigated in recent decades as a result of their potential for health-related benefits to humans (Yerramsetty et al. 2011).

The biosynthesis of several secondary metabolites in plants is widely believed to be a part of the plants defense responses to stress (Xu et al. 2012). Usually, the production of secondary metabolites stimulated by environmental stress is mediated by signaling compounds. Tossi et al. (2009) revealed NO induction by UV-B radiation using the fluorophore 4,5-diamino-fluorescein diacetate(DAF-2DA). In our previous study, we have confirmed that nitric oxide (NO), as an essential signaling molecule, mediated isoflavone synthesis under UV-B stress (Jiao et al. 2016b). NO burst is an early response of plants to UV-B radiation (Zhang et al. 2011). The signaling transduction of NO usually needs the involvement of downstream signaling molecules.

Accordingly, in airway smooth muscle, NO donors, sodium nitroprusside (SNP) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) regulated ADP-ribosyl cyclase (ADPRC) activity (White et al. 2002). This indicates NO might be capable of affecting cADPR production. 8-Br-cADPR (8-Bromo-cyclic ADP-ribose), the

antagonist of cADPR, weakened S-nitroso-L-glutathione (GSNO) and SNAP-induced pathogenesis-related 1 protein (PR-1) activation in tobacco, demonstrating that the activation by NO of plant defense system is, at least in part, dependent upon cADPR signaling pathway (Klessig et al. 2000). In addition, exogenous supplementation with cADPR could induce phenylalanine ammonia lyase (PAL) and PR-1 expression in tobacco (Wendehenne et al. 2001). Our previous study further authenticated that cADPR acts as a secondary messenger to mediate abscisic acid (ABA)-induced isoflavone production in soybean sprouts (Jiao et al. 2016a). Nevertheless, little is known about a causal relationship between cADPR signaling pathway and NO-induced isoflavone accumulation under UV-B radiation.

Signaling transduction of NO usually needs the involvement of guanosine 3',5'-cyclic monophosphate (cGMP), a downstream signaling molecule. NO can elevate cGMP levels in plant tissues (Dubovskaya et al. 2011). It has been reported that cGMP could activate cADPR synthesis in the sea urchin egg (Wilson and Galione 1998), thereby serving as a further downstream signal molecular of NO. cGMP stimulates the cADPR biosynthesis from beta-NAD⁺ through activating its synthetic-enzyme ADPRC in sea urchin eggs and egg homogenates, and cADPR may transduce signals generated by cell surface receptors or gaseous transmitters linked to cGMP production (Galione et al. 1993). This suggests that cGMP regulates the cADPR synthesizing activity more closely related to the ADPRC in sea urchin eggs. Whether cGMP is involved in endogenous NO- mediated ADPRC/cADPR pathway in soybean sprouts under UV-B radiation, remains to be proven. In our previous study,

we confirmed that cGMP acts as a second messenger of NO, to mediate UV-B radiation-stimulated isoflavone accumulation in soybean sprouts (Supplementary Figure S1). The role of cADPR in NO-cGMP-induced isoflavone accumulation in soybean sprouts under UV-B radiation should be deeply investigated.

The objectives of the present study are to explore the role of NO/cGMP/cADPR pathway in UV-B stress-stimulated isoflavone synthesis in soybean sprouts and to explore relevant enzymatic and molecular mechanisms.

Materials and methods

Plant material, growth conditions and experimental design

The washed soybean seeds (*Glycine max* L. cv *Yunhe*) were soaked with distilled water at 30 °C for 8 h, and then spread evenly in a sprouter. Temperature for germination was set as 30 °C.

For UV-B treatment, a 15 W UV light bulb (ranging from 280 to 320 nm with a peak at 313 nm; radiation intensity 40 $\mu\text{W}\cdot\text{cm}^{-2}$; Beijing Electronic Resource, Inc., Beijing, China) was installed at a distance of 35 cm above the sprouter inside the chamber. Chemical reagents used for treating soybean sprouts were manufacturer's instructions, and given the final concentrations of 0.5 mM SNP, 0.5 mM cPTIO, 10 μM LY83583, 50 μM 8-Br-cGMP, 1 mM nicotinamide. Controls (CK) were sprayed with as follows: 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was used as a scavenger of NO, SNP as a NO donor, 8-Br-cGMP as a cGMP analog, 6-Anilino-5,8-quinolinequinone (LY-83583) as an inhibitor of GC, and nicotinamide as an inhibitor of ADPRC. All chemical reagents were purchased from

Sigma-Aldrich (St. Louis, MO, USA). These reagents were dissolved with distilled water or 0.2% dimethyl sulfoxide (DMSO) per distilled water only. Soybean seeds were treated with the reagents or continuous UV-B radiation plus a reagent for 4 days. Sprouts were treated with the reagents three times daily (at 8:00, 16:00 and 24:00).

The sampled soybean sprouts used for isoflavone content determination were lyophilized using a Labconco freeze-dryer. Dried sprouts were ground to fine powder, which then was stored at -20 °C. For the other assays, the sampled fresh sprouts were immediately frozen and stored at -80 °C.

Determination of isoflavone content

The ground soybean sample was accurately weighed for 0.20 g and extracted with 6 mL of 80% methanol by ultrasonic-assisted method at 40 °C for 30 min, and then centrifuged. The supernatant was collected. Isoflavone content was analyzed by high performance liquid chromatography (HPLC) as described by Jiao et al. (2016b).

Measurement of isoflavone biosynthetic-enzymes activity

Activity of chalcone synthase (CHS) was detected by the CHS assay system kit (GE Healthcare, Shanghai, China) per corresponding protocol.

Activity of isoflavone synthase (IFS) was analyzed as described by Kochs and Grisebach et al. (1986) and calculated by integration of the peak areas of genistein and naringenin.

Measurement of cADPR content

Freshly harvested soybean sample was immediately frozen in liquid nitrogen. Frozen soybean sample was pulverized into a fine powder with liquid nitrogen. The

frozen powder was extracted in 0.8 volumes of ice-cold 0.6 M perchloric acid, and then incubated on ice for 20 min. Extraction, treatment with hydrolytic enzymes and purification of cADPR was according to Graeff and Lee (2002) except that cADPR was further purified by chromatography as described by Da Silva et al. (1998). Radioimmunoassays were carried out according to Graeff et al. (1997) using antibodies against cADPR (Chemicon International, Inc., Temecula, CA, USA).

Assay of ADPRC activity

Freshly harvested soybean sample was rapidly frozen in liquid nitrogen. Frozen soybean sample was pulverized into a fine powder with liquid nitrogen. To assay ADPRC activity, the ground powder was re-suspended in buffer containing 40 mM HEPES (pH 7.0), 100 mM NaCl, 5mM MgCl₂, 4% glycerol, 0.05% NP-40, 10mM NaF, 20mM β -glycerolphosphate (freshly supplemented with 2 mM sodium vanadate), 5mM DTT, 1 mM PMSF, 30 nM okadaic acid, and complete EDTA-free protease inhibitors (Roche, Palo Alto, CA, USA). Re-suspended material was sonicated for 20 sec, and then centrifuged at 12,000g for 10 min. The collected supernatant was then centrifuged at 100,000g for 1 h. The membrane pellet was re-suspended in 15% glycerol, 0.02% TritonX-100 and divided into aliquots. ADPRC activity was also assayed by HPLC analysis (Da Silva et al. 1998).

Gene expression assay (Quantitative Real-Time PCR, QRT-PCR)

Total RNA from 3 separate batches of soybean sprouts was extracted using an E.Z.N.A.[™] Plant RNA Kit (Omega, Norcross, GA, USA; R6827-01) according to corresponding protocol. First-strand cDNA was synthesized as described by Jiao et al.

(2016b). PCR amplification was carried out using the TaKaRa *Ex-Taq*TM polymerase for target genes and *EF1b*. For QRT-PCR assays, the primers used in this study are listed in Table 1. QRT-PCR analysis was carried out with the SYBR[®] *Premix Ex Taq*TM (TAKARA: RR420A) in the ABI 7500 sequence detection system per corresponding protocol (Applied Biosystems, Foster City, CA, USA). The PCR cycling conditions were: 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s, and then 60 °C for 30 s. Each determination was carried out with 3 independent biological replicate experiments. For each experiment, 3 technical replicates were performed.

Western blot

Sprouts tissue used for Western blot assays was sampled on day 4. Tissue lysates were obtained using RIPA buffer containing a protease inhibitor cocktail. The mixtures were then centrifuged. The 12 µL samples containing 40 µg of protein each were loaded into a 10% (w/v) SDS-PAGE gel at 80 volts for 2 hours, and then transferred to a 0.45 mm polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA). Subsequently, membranes were blocked for 1 hour at room temperature with 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline with 0.1% Tween 20 (TBST), washed in TBST, and then incubated with primary antibody (anti-PI-PLC, anti-CHS and anti-IFS) overnight at 4 °C, followed by incubation with secondary goat polyclonal antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG, 1:5000, Bio-Rad; mouse anti-rabbit IgG, 1:5000, Merck Millipore Darmstadt, Germany) for 1 hour at room temperature. Membranes were washed 9

times for 5 min each with TBST. Anti-rubisco antibody was used to normalize. The SuperSignal® West Dura Extended Duration ECL Substrate (Bio-Rad, Warsaw, Poland) was added to measure immunocomplexes per corresponding protocol, which were then visualized with an X-ray film system. BandScan 5.0 software was used to quantify the relative levels of immunoreactivity.

Statistical analyses

Experimental data were expressed as the mean $\pm$ standard deviation (SD) with three replicates ($n=3$). SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was employed for the significant difference test. Variables of three replicates were compared using standard ANOVA procedure. Significance was indicated at a p value of 5% or less.

Results

NO-cGMP-induced cADPR increase in soybean sprouts under UV-B exposure

During 4 days of germination, compared with the control, cADPR level (Fig. 1A) and ADPRC activity (Fig. 1B) in soybean sprouts under UV-B radiation were significantly higher ($p < 0.05$). cPTIO, a specific NO-scavenger, significantly weakened the increase in cADPR level (Fig. 1A) and ADPRC activity (Fig. 1B) in soybean sprouts induced by UV-B radiation ($p < 0.05$). Exogenous NO donor, SNP application significantly reversed the impact from cPTIO on cADPR level (Fig. 1A) and ADPRC activity (Fig. 1B) ($p < 0.05$). Compared with the control, supplementation of SNP used alone could also significantly enhance cADPR level (Fig. 1A) and ADPRC activity (Fig. 1B) ($p < 0.05$).

Furthermore, LY83583 significantly hindered UV-B-induced cADPR level (Fig. 2A) and ADPRC activity (Fig. 2B) during 4 days of germination ($p < 0.05$). Application of 8-Br-cGMP and SNP could significantly reverse the decrease caused by LY83583 in cADPR level (Fig. 2A) and ADPRC activity (Fig. 2B) ($p < 0.05$). In addition, compared with the control, the application of exogenous 8-Br-cGMP and SNP used alone could significantly up regulate cADPR level (Fig. 2A) and ADPRC activity (Fig. 2B) ($p < 0.05$).

Effects of NO-cGMP-induced cADPR on isoflavone accumulation under UV-B stress in soybean sprouts.

To further investigate whether NO-cGMP-triggered cADPR synthesis was involved in isoflavone accumulation, we explored the effects of nicotinamide on UV-B stress-stimulated isoflavone accumulation. Our results showed that during germination, application of nicotinamide significantly decreased UV-B stress-stimulated cADPR content (Fig. 3A), isoflavone production (Fig. 3B), activity of CHS (Fig. 3C) and IFS (Fig. 3D) as well as the gene expression of CHS (Fig. 3E) and IFS (Fig. 3F) stimulated by UV-B stress ($p < 0.05$). Exogenous SNP and 8-Br-cGMP could significantly reverse the above decrease caused by nicotinamide (Fig. 3) ($p < 0.05$). Compared with the control, the application of exogenous SNP and 8-Br-cGMP used alone could also significantly up regulate cADPR content (Fig. 3A), isoflavone production (Fig. 3B), activity of CHS (Fig. 3C) and IFS (Fig. 3D) as well as the gene expression of CHS (Fig. 3E) and IFS (Fig. 3F) ($p < 0.05$).

Compared with the control, UV-B radiation significantly induced elevation of

protein expression of CHS and IFS in 4-day-old soybean sprouts ($p < 0.05$) (Fig. 4). Nicotinamide significantly weakened the positive effects of UV-B stress on protein expression of CHS and IFS ($p < 0.05$) (Fig. 4). In addition, compared with the control, the application of SNP (exogenous NO donor) and 8-Br-cGMP (cGMP analog) used alone could also significantly up regulate protein expression of CHS and IFS ($p < 0.05$) (Fig. 4).

Discussion

Compared with the control, UV-B stress significantly stimulated cADPR production in soybean sprouts (Fig. 1A). This was attributed to the increase in ADPRC activity (Fig. 1B). However, cPTIO weakened the UV-B radiation-activated elevation in cADPR production (Fig. 1A) and ADPRC activity (Fig. 1B). The attenuation can be reversed by supplementation with SNP (exogenous NO donor) (Fig. 1). Compared with the control, SNP used alone can also significantly elevate cADPR production (Fig. 1A) and ADPRC activity (Fig. 1B). These results suggest that NO is an essential signal for mediating UV-B radiation-activated cADPR synthesis in soybean sprouts. UV-B, an external stress, is not directly involved in plant growth and development, instead it activates its corresponding effectors like NO. Subsequently, NO can facilitate the transduction of UV-B stress signal to a series of downstream defense reactions (Eilert 1987), like cADPR synthesis (Fig. 1A) and ADPRC activation (Fig. 1B). The accumulation of cADPR might be due to the enhancement of ADPRC activity (Sánchez et al. 2004). UV-B-induced NO stimulated ADPRC probably via a protein kinase A (PKA)-mediated phosphorylation (Scarfi et al. 2009).

The increase of ADPRC activity might be attributed to over expression of genes and proteins of ADPRC under stress (Sánchez et al. 2004), which then converted NAD to the intracellular cADPR (Scarfi et al. 2008).

In plants, NO biosynthesis is an early response to UV-B radiation (Tossi et al. 2009). Signal transduction of NO usually requires the involvement of downstream signaling molecules. We further investigated the effects of cGMP on cADPR synthesis in this study. Our data showed that the enhancement of cADPR accumulation (Fig. 2A) and ADPRC activity (Fig. 2B) induced by UV-B stress was partly inhibited by LY83583. 8-Br-cGMP and SNP reversed the negative impact from LY83583 (Fig. 2). cADPR accumulation (Fig. 2A) and ADPRC activity (Fig. 2B) in the soybean sprouts treated with both 8-Br-cGMP and SNP were much higher than those of the control. Hence, it could be concluded that cADPR accumulation stimulated by UV-B radiation was mediated by cGMP. cGMP induces the cADPR biosynthesis via activating its biosynthetic-enzyme ADPRC (Galione et al. 1993). Accordingly, the role of cGMP in activating cADPR production may be via a cGMP-dependent protein kinase (Rah et al. 2005). In summary, in view of our previous results that NO could induce cGMP accumulation (Supplementary Figure S1), we could infer that cGMP pathway mediates NO-stimulated cADPR synthesis in soybean sprouts under UV-B radiation.

In our previous study, we have confirmed that NO, with cGMP as a second messenger, stimulated isoflavone accumulation under UV-B radiation (Supplementary Figure S1). To further investigate whether cADPR was involved in NO-cGMP

pathway-induced isoflavone production, we explored the effects of UV-B radiation on isoflavone synthesis, activity and expression of CHS and IFS in soybean sprouts in the presence of nicotinamide. The results showed that treatment with nicotinamide significantly suppressed cADPR content (Fig. 3A), isoflavone production (Fig. 3B), activity of CHS (Fig. 3C) and IFS (Fig. 3D) as well as the gene expression of CHS (Fig. 3E) and IFS (Fig. 3F) under UV-B treatment. Moreover, the data also exhibited the reversion by SNP and 8-Br-cGMP on nicotinamide-caused inhibition. Supplementation with SNP and 8-Br-cGMP also had positive effects on cADPR production (Fig. 3A), isoflavone production (Fig. 3B), activity of CHS (Fig. 3C) and IFS (Fig. 3D) as well as the gene expression of CHS (Fig. 3E) and IFS (Fig. 3F). Hence, it could be concluded that isoflavone synthesis enhanced by UV-B-triggered NO-cGMP was, at least in part, mediated by cADPR. The induction of isoflavone accumulation by cADPR might be mediated by Ca^{2+} . cADPR might interact positively with free Ca^{2+} to shape cytosolic free Ca^{2+} oscillations whose frequency and amplitude, in turn, would be tightly regulated (Leckie et al. 1998). Thus, cADPR is a plant mediator of Ca^{2+} mobilization. A rise of $[\text{Ca}^{2+}]_i$ evoked by UV-B radiation could then elevate expression of isoflavone biosynthetic-enzymes by activation of their promoters (Frohnmeier et al. 1999). Overall, NO-cGMP-induced cADPR enhanced isoflavone accumulation by activating the expression of protein and gene for CHS and IFS in soybean sprouts under UV-B stress.

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Figure Captions

Fig. 1. Effects of UV-B-triggered NO on cADPR content (A) and ADPRC activity (B) in soybean sprouts. Values are the mean $\pm$ SD (error bars) with three replicates ($n=3$). The different letters represent the significant differences among various treatments ($p < 0.05$).

Fig. 2. Effects of cGMP on cADPR content (A) and ADPRC activity (B) in soybean sprouts. Values are the mean $\pm$ SD (error bars) with three replicates ($n=3$). The different letters represent the significant differences among various treatments ($p < 0.05$).

Fig. 3. Effects of ADPRC inhibitor on IP₃ production (A), isoflavone content (B), activity (C, D) and gene expression (E, F) of CHS and IFS in soybean sprouts. Values are the mean $\pm$ SD (error bars) with three replicates ($n=3$). The different letters represent the significant differences among various treatments ($p < 0.05$).

Fig. 4. Effects of ADPRC inhibitor on protein expression of isoflavone biosynthetic-enzymes by Western blot analysis. Panels show representative bands (A). Histograms represent relative protein levels of CHS (B) and IFS (C) of 4-day-old soybean sprouts normalized to the corresponding rubisco. Values are the mean $\pm$ SD (error bars) with three replicates ($n=3$). The different letters represent the significant differences among various treatments ($p < 0.05$).

Figure S1. Effects of cGMP inhibitor on cGMP (A) and isoflavone (B) content of soybean sprouts under UV-B stress. Values are the mean $\pm$ SD (error bars) with three replicates ($n=3$). The different letters represent the significant differences among various treatments ($p < 0.05$).

Tables

Table 1. Sequence-specific primers used in this study.

Gene	Primer name	Primer sequences (5'→3')
<i>CHS</i>	Sense	GCTTGTTGTCTGTTCTGAG
	Ant-sense	CACCTTCACTGTCTGGAG
<i>IFS</i>	Sense	TGGAAGTTCGTGAGGAAG
	Ant-sense	ATGGAGATGGTGCTGTTG
<i>EF1b</i>	Sense	CCACTGCTGAAGAAGATGATGATG
	Ant-sense	AAGGACAGAAGACTTGCCACTC

Fig. 1

Fig. 2

Fig. 3

Fig. 4