Canadian Journal of Plant Science
Revue canadienne de phytotechnie

Mitogen-activated protein kinase mediates nitric oxide-induced isoflavone accumulation in soybean sprouts under UV-B radiation

Journal:	<i>Canadian Journal of Plant Science</i>
Manuscript ID	CJPS-2016-0369.R2
Manuscript Type:	Article
Date Submitted by the Author:	21-Mar-2017
Complete List of Authors:	Jiao, Caifeng Yang, Runqiang Wang, Pei; Nanjing Agricultural University Tian, Lu; Nanjing Agricultural University Gu, Zhenxin
Keywords:	MAPK, UV-B, NO, isoflavone, iTRAQ analysis

SCHOLARONE™
Manuscripts

**Mitogen-activated protein kinase mediates nitric oxide-induced isoflavone
accumulation in soybean sprouts under UV-B radiation**

Caifeng Jiao, Runqiang Yang, Pei Wang, Lu Tian, Zhenxin Gu *

College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095,

People's Republic of China

Corresponding Author

E-mail: guzx@njau.edu.cn

For Review Only

Abstract. Mitogen-activated protein kinase (MAPK) cascades are critical components of signal transduction pathways. However, little is known about whether MAPK is involved in phenylpropanoid pathway under UV-B radiation. In this study, we investigated the role of MAPK in mediating nitric oxide (NO)-induced isoflavone accumulation in soybean sprouts under UV-B radiation. UV-B-triggered NO stimulated gene and protein expression of MAPK. Furthermore, U0126 [1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene] (MAPKK inhibitor) hindered UV-B-induced isoflavone accumulation, gene and protein expression of enzymes involving in isoflavone biosynthesis (chalcone synthase, CHS; isoflavone synthase, IFS). The attenuation was reversed by exogenous NO donor sodium nitroprusside (SNP). SNP treatment used alone could also enhance isoflavone synthesis, gene and protein expression of CHS and IFS. Overall, MAPK mediates NO-induced isoflavone accumulation in soybean sprouts under UV-B radiation.

Key words : MAPK; UV-B; NO; isoflavone; iTRAQ analysis

Abbreviations

MAPK, mitogen-activated protein kinase; CHS, chalcone synthase; IFS, isoflavone synthase; U0126, 1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene; SNP, sodium nitroprusside; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; NO, nitric oxide; iTRAQ, isobaric tag for relative and absolute quantitation.

Introduction

Isoflavones are a class of molecules called flavonoids that belong to a large family of polyphenols and synthesized predominantly in legume. Due to their role in plant defense (Masilamani et al. 2012) and health-related benefits for human (Du et al. 2010), the metabolism and accumulation of isoflavonoids in plants were investigated in recent years.

Accumulation of many secondary metabolites in plants is part of their defense responses to environmental cues and/or stress (Hahlbrock et al. 2003). Several secondary metabolites are related to plant defense responses, though they may be not directly involved in plant growth and development. Plants may enhance their resistance by inducing secondary metabolite biosynthesis under stresses (Xu et al. 2012). Usually, the induction of these secondary metabolites by exogenous stresses is mediated by endogenous signaling systems (Xu et al. 2012). Recently, we reported that NO, as an essential signaling molecule, mediates UV-B radiation-induced isoflavone accumulation in soybean sprouts (Jiao et al. 2016). Nevertheless, signal transduction of NO usually needs the involvement of downstream signaling molecules.

Several lines of evidence from biochemical and genetic studies of plant stress signaling indicate that reversible protein phosphorylation plays an important role in the regulation of physiological status and gene expression in response to various environmental stresses (Xiong and Yang 2003; Yuasa et al. 2001). MAPK cascade is believed to be one of the major pathways by which extracellular stimuli are transduced into intracellular responses in plant cells (Nakagami et al. 2005; Zhang et al. 2007). This cascade consists of a set of three functionally interlinked kinases: MAPK and immediate upstream activators, MAPK kinase and MAP kinase kinase kinase (Pan et al. 2008). It is documented that UV-B radiation could activate MAPK (Holley et al. 2003; Ramani and

Chelliah 2006). In addition, it has been shown that NO is involved in the activation of MAPK activity during the plant defense responses against pathogen infections in tobacco (Kumar and Klessig 2000) and *Arabidopsis thaliana* (Clarke et al. 2000), and the adventitious root formation induced by indole acetic acid in cucumber (Pagnussat et al. 2004). It is not clear whether MAPK is induced by endogenous NO and involved in defense responses under UV-B stress.

Accordingly, MAPK play significant roles in UV-B signaling leading to stimulation of tryptophan decarboxylase (Tdc) and strictosidine synthase (Str) genes and the accumulation of catharanthine in *C. roseus* cell suspension cultures (Ramani and Chelliah 2006), implying that the MAPK signal pathway(s) was related to secondary metabolism. Both of MAPK and isoflavones accumulation are the results of stress stimuli. However, reports on the involvement of MAPK in endogenous NO-induced isoflavone biosynthesis of soybean sprouts under UV-B radiation are not available in the literature.

This study investigated the mediation of isoflavone accumulation by endogenous NO-induced MAPK in soybean sprouts under UV-B radiation and explored the mechanisms of UV-B stress-elicited isoflavone accumulation.

Materials and Methods

Plant material, growth conditions and experimental design

Clean 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, during germination, a 15 W UV-B light bulb (central wavelength 313 nm; radiation intensity $40 \mu\text{W}\cdot\text{cm}^{-2}$) was placed at a distance of 1 ft above the sprouter in the chamber. The chemical compounds used for soybean sprouts treatment were purchased from Sigma-Aldrich

(St. Louis, MO, USA). SNP was used as an exogenous NO donor, cPTIO as a NO scavenger, U0126 as a MAPKK inhibitor. These reagents were used at the final concentrations of 0.5 mM SNP, 0.5 mM cPTIO and 5 μ M U0126. The controls were sprayed with distilled water only. Soybean seeds were exposed to these reagents or to UV-B radiation plus chemical reagents for 4 days. The soybean sprouts were sprayed with these 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 a fine powder, which was then stored at -20 °C. For the other assays, fresh sprouts were immediately frozen and stored at -80 °C.

Isoflavone content analysis

The ground soybean sample was accurately weighed (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. (2016).

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

Total RNA from soybean sprouts was isolated by a E.Z.N.A.TM Plant RNA Kit (OMEGA, R6827-01). First-strand cDNA was synthesized as described by Jiao et al. (2016). PCR amplification was performed using TaKaRa *Ex-Taq*TM polymerase for target genes and internal reference (*EF1b*). Table 1 lists sequence-specific primers for QRT-PCR analysis. Triplicate quantitative assays were performed on each cDNA with the SYBR[®] *Premix Ex Taq*TM (TAKARA: RR420A) in ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). The PCR process included 1 cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s.

Western blot

Tissue for Western blot analysis was collected on day 4. Tissue lysates were collected in RIPA buffer with a protease inhibitor cocktail. Then, the reaction mixtures were cleared by centrifugation ($14,000 \times g$ for 30 min at 4 °C). Samples containing 40 µg of protein each, were loaded into a 10% (w/v) SDS-PAGE gel and transferred to a 0.45 mm polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts, USA). Subsequently, the membranes were blocked for 1 h at room temperature using 5% non-fat dry milk (Bio-Rad) in Tris-buffered saline with 0.1% Tween 20 (TBST), washed in TBST, incubated overnight at 4 °C with primary antibody (anti-MAPK, anti-CHS, anti-IFS) and incubated for 1 h at room temperature with a 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). The membranes were washed 4 times for 5 min each and 5 times for 5 min each with TBST. Anti-rubisco antibody was used to normalize for protein loading. The SuperSignal® West Dura Extended Duration ECL Substrate (Bio-Rad, Warsaw, Poland) was used to detect immunocomplexes, which were then visualized using an X-ray film system. The BandScan 5.0 software was used to quantify the relative levels of immunoreactivity.

Protein extraction and iTRAQ labeling

Protein extracts of 4-day-old soybean sprouts were obtained by using the P-PER™ Plant Protein Extraction Kit (89803, Thermo Fisher Scientific, CA, USA) according to the manufacturer's recommendation. The extracted protein samples (250 µg each) were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested using Filtered aide sample preparation (FASP) method by sequencing grade trypsin (V5111, Promega, CA, USA). After trypsin digestion, peptides

were dried by vacuum centrifugation and reconstituted in iTRAQ dissolution buffer and labeled using iTRAQ 8-plex kits (AB Sciex Inc., MA, USA) according to the manufacturer's protocol. Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 70 μ L isopropanol. Control and UV-B treatment samples were labeled with 113 and 114 tags, respectively. Three biological replicates were conducted. After labeling and quenching, the samples were combined and dried by vacuum centrifugation.

Fractionation by High pH RP chromatography

High pH RP chromatography was performed with an UltiMate™ 3000 HPLC Pump system (Thermo Fisher Scientific, CA, USA). The iTRAQ labeled peptide mixtures were reconstituted with RP buffer A (98% H₂O, 2% ACN, pH 10.0) and loaded onto a 2.1×100 mm ACQUITY UPLC BEH C18 column containing 1.7 μ m particles (Waters, MA, USA). The peptides were eluted with a flow rate of 0.2 mL/min with a gradient of 97% RP buffer A for 12 min, 3-98% RP buffer B (98 % ACN, 2% H₂O, pH 10.0) for 40 min. The system was then maintained in 98% RP buffer B for 15 min before equilibrating with RP buffer A for 10 min prior to the next injection. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 12 fractions and vacuum-dried.

MS/MS analysis by LTQ-Orbitrap XL

Each fraction was reconstituted in buffer A [2% ACN, 0.1% formic acid (FA)] and centrifuged at 20,000 ×g for 10 min. Using an auto sampler, 20 μ L of supernatant was loaded onto a 2 cm C18 trap column (inner diameter 200 μ m) on a nano HPLC. Peptides were eluted onto a resolving 10 cm analytical C18 column (inner diameter 75 μ m) that was assembled in-house. The samples were loaded at 4 μ L/min for 8 min and eluted with a 88 min gradient at 400 nL/min from 3 to 28% B

(98 % ACN, 0.1% FA), 20 min from 28 to 55% B and followed by a 5 min linear gradient to 98% B, maintenance at 98% B for 20 min, and finally a return to 3% B over 1 min. The peptides were subjected to nano electrospray ionization followed by tandem mass spectrometry (MS/MS) in an LTQ-Orbitrap XL (ThermoFisher Scientific, CA, USA) coupled online to the HPLC. Intact peptides were detected in the Orbitrap with a resolution of 60, 000. Peptides were selected for MS/MS using high-energy collision dissociation (HCD) operating mode with a normalized collision energy setting of 40.0; ion fragments were detected in the Orbitrap at a resolution of 7500. A data-dependent procedure that alternated between one MS scan followed by 5 MS/MS scans was applied for the 5 most abundant precursor ions above a threshold ion count of 5000 in the MS survey scan with duration of 60 s. The electrospray voltage applied was 2.4 kV. Automatic gain control (AGC) was used to optimize the spectra generated by the orbitrap. The AGC target for full MS was 1^6 and 1^5 for MS2. For MS scans, the m/z scan range was 350 to 1800 Da. For MS2 scans, the m/z scan range was 100-1800.

Database search and iTRAQ quantification

Samples were analyzed by nano-LC-MS/MS. Generation of peak lists was performed with the Proteome Discoverer 1.3 (Thermo Fisher Scientific, CA, USA). Protein identification was performed using SEQUEST search engine against the NCBI *Glycine* max database (Apr 20th, 2015). For protein identification, a mass tolerance of 10 ppm was permitted for intact peptide masses and 0.02 Da for fragmented ions, with allowance for one missed cleavages in the trypsin digests. Oxidation at methionine was set as the potential variable modifications, while carbamidomethylation at cysteine was set as fixed modifications. The charge states of peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in SEQUEST by

choosing the decoy checkbox in which a random sequence of database is generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, only peptides at the 99% confidence interval by a SEQUEST probability analysis were counted as identified and each identified protein includes at least one unique peptide. Only proteins fold change of >1.5 were considered.

Bioinformatic analysis of proteins

Functional annotations were performed with Blast2GO software (<http://www.geneontology.org>) against the non-redundant protein database and Clusters of Orthologous Groups of Proteins System (COG) software (<http://www.ncbi.nlm.nih.gov/COG/>). To take advantage of the current knowledge of biochemical pathways and other types of molecular interactions, we used KEGG databases (<http://www.genome.jp/kegg/pathway.html>).

Statistical analyses

Experimental data were expressed as the mean $\pm$ standard deviation (SD) of three replicates. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was applied for significant difference test. Variables of three replicates were compared using standard ANOVA procedure followed by Duncan test. Statistically significant difference was accepted at a level of $p < 0.05$.

Results

Induction of MAPK by endogenous NO in soybean sprouts under UV-B radiation

Gene (Fig. 1) and protein (Fig. 2) expression levels of *MAPK* in the soybean sprouts treated with UV-B radiation were much higher than those of the control, indicating that UV-B exposure activated MAPK. The highest gene expression level of MAPK was observed on day 4 after UV-B treatment, which was 1.8 times higher than that of the control (Fig. 1). For 4-day-old soybean

sprouts, protein expression level of MAPK under UV-B treatment was 1.4 times higher than that of the control (Fig. 2). In addition, the positive effects of UV-B radiation on the gene and protein expression of MAPK were inhibited by cPTIO (NO inhibitor) by 21% and 19% in 4-day-old soybean sprouts, respectively, while the inhibition was reversed by the application of SNP (NO donor) (Fig. 1 and 2). SNP treatment used alone could also enhance gene and protein expression of MAPK (Fig. 1 and 2). The highest gene expression level of MAPK under SNP treatment was obtained on day 4, which was 1.9 times higher than that of the control (Fig. 1). Protein expression level of MAPK under SNP treatment in 4-day-old soybean sprouts was 2.0 times higher than that of the control (Fig. 2). Hence, it could be concluded that MAPK expression in soybean sprouts was truly induced by NO under UV-B radiation.

Mediation of isoflavone accumulation by NO-induced MAPK under UV-B radiation

UV-B radiation significantly induced increase in isoflavone content (Fig. 3A), gene (Fig. 3B and 3C) and protein (Fig. 4) expression of isoflavone biosynthetic-enzymes (CHS and IFS) in the soybean sprouts. The highest isoflavone content and gene expression levels of CHS and IFS were observed on day 4 after UV-B treatment, which were 1.7, 1.6 and 1.5 times higher than those of the control, respectively (Fig. 3). For 4-day-old soybean sprouts, protein expression levels of CHS and IFS under UV-B treatment were 1.7 and 1.8 times higher than those of the control, respectively (Fig. 4). In addition, U0126 (MAPKK inhibitor) inhibited UV-B radiation-induced isoflavone content and gene expression levels of CHS and IFS by 20%, 19% and 31% in 4-day-old soybean sprouts respectively (Fig. 3). For 4-day-old soybean sprouts, protein expression levels of CHS and IFS decreased by U0126 by 32% and 34%, respectively (Fig. 4). The inhibition was reversed by the application of SNP (Fig. 3 and 4). SNP treatment used alone could also enhance isoflavone

accumulation (Fig. 3), gene (Fig. 3B and 3C) and protein (Fig. 4) expression of CHS and IFS. Isoflavone content and gene expression levels of CHS and IFS increased under SNP treatment, which were 2.1, 2.3 and 2.0 times higher than those of the control, respectively (Fig. 3). Protein expression levels of CHS and IFS under SNP treatment in 4-day-old soybean sprouts were 2.5 and 3.7 times higher than those of the control (Fig. 4). Therefore, the data demonstrated that NO-induced MAPK mediated the enhancement of isoflavone accumulation by up regulating genes and proteins expression of isoflavone biosynthetic-enzymes in soybean sprouts under UV-B radiation.

Proteins expression after UV-B and SNP treatments through iTRAQ analysis

To reveal molecular mechanism of UV-B signaling-induced isoflavone accumulation, iTRAQ technique was used to study the proteomic changes. The detailed protein identifications and quantitations are listed in Supplemental Table S1 and S2. A summary of the results of the protein related to signal transduction is presented in Table 2. Both of UV-B and SNP treatments induced expression of MAPKK (gi:356534821) and calreticulin (gi:356531872). Furthermore, protein expression of isoflavone biosynthetic-enzymes (CHS (gi:571505833) and IFS (gi:7288453)) were also up-regulated in response to UV-B and SNP treatments.

Discussion

In our previous study, we have confirmed that NO could induce isoflavone accumulation under UV-B radiation (Jiao et al. 2016). Plant defense responses are usually mediated by complex signaling network. To investigate the signaling cascade mechanisms of UV-B-induced isoflavone accumulation, we examined the effects of UV-B treatment on MAPK expression. In the present work, UV-B treatment induced increase in gene (Fig. 1) and protein (Fig. 2) expression of MAPK.

The enhancements were blocked by the treatment with the NO inhibitor, cPTIO (Fig. 1 and 2), While the inhibition could be reversed by NO donor SNP. Moreover, treatment with the SNP alone also induced increases in gene (Fig. 1) and protein (Fig. 2) expression of MAPK. The results suggested that NO could activate MAPK under UV-B radiation. Accordingly, NO treatment of tobacco induced a significant increase in salicylic acid (SA) levels (Durner et al. 1998). The ability of NO to activate MAPK was suppressed in transgenic NahG tobacco, which are unable to accumulate SA (Kumar and Klessig 2000), suggesting that NO induction of MAPK may utilize an SA-dependent signaling pathway. Furthermore, as seen from iTRAQ analysis, both of UV-B and SNP treatments induced calreticulin expression (Table 2). Accordingly, evidence was obtained that the activation of the tobacco MAPK SIPK (Salicylic acid-induced protein kinase) by NO donors required a transient influx of extracellular Ca^{2+} in tobacco cells (Courtois et al. 2008). Therefore, we could speculate that calreticulin might act as a downstream messenger of NO to activate MAPK under UV-B radiation. In addition, in response to UV-B and SNP treatments, expression of MAP kinase kinase (MAPKK) also elevated (Table 2), which then activates a MAPK (Pan et al. 2008). This final kinase phosphorylates nuclear target proteins to activate appropriate gene expression (Bayram et al. 2012; Román et al. 2007; Saito 2010). Thus, it could be inferred that UV-B-induced NO, with calreticulin as a second messenger, elicited protein expression of MAPKK, followed by activating MAPK protein, leading to induction of gene expression of MAPK.

MAPK cascades are critical downstream components of signal transduction pathways in eukaryotic organisms (Singh et al. 1997). A previous study has reported that MAPK played important roles in UV-B signaling-induced accumulation of catharanthine (Ramani and Chelliah 2006). However, little is known about whether MAPK is involved in phenylpropanoid pathway

under UV-B radiation. So we further investigate the role of NO-induced MAPK in isoflavone accumulation under UV-B radiation. Our data suggested that NO-triggered MAPK induced isoflavone production in the soybean sprouts under UV-B radiation (Fig. 3A). This resulted from up-regulation of gene (Fig. 3B and 3C) and protein (Fig. 4) (Table 2) expression of isoflavone biosynthetic-enzymes (CHS and IFS). UV-B radiation, an external stressful environmental signal, is not directly involved in the secondary metabolism in plants, instead it activates downstream mediators (Eilert 1987). The MAPK transduction cascades are important mediators in signal transmission, connecting the perception of external stimuli to cellular responses (Pagnussat et al. 2004). The activated MAPK by stress could phosphorylate various stress-related proteins to induce cellular defense responses (Neill et al. 2002), including secondary metabolite production (Morris 2001; Ramani et al. 2010). The activation of phenylpropanoid pathway as a response to a wide diversity of stress-factors was identified as a genetic marker for the induction of plant defense responses (Pellinen et al. 2002). MAPK generated in plants acts as local and/or systemic signal to activate a series of defense reactions (Morris 2001), such as induction of proteins synthesis for limit of stress damage (Table 2) (Fig. 4) and gene expression (Fig. 3B and 3C) of key enzymes (CHS and IFS) participating in isoflavone synthesis and accumulation (Fig. 3A) .

Conclusions

MAPK activated by NO mediates enhancement of gene and protein expression of CHS and IFS, leading to elevation of isoflavone accumulation under UV-B radiation. This work makes a foundation upon which to build a research program that can make a contribution to plant signaling and has practical implications in commercial production of functional foods.

Acknowledgment

This project was supported by the National Natural Science Foundation of China (31671862).

References

- Bayram, Ö., Bayram, Ö. S., Ahmed, Y. L., Maruyama, J., Valerius, O., Rizzoli, S. O., Ficner, R., Irniger, S. and Braus, G. H. 2012. The *Aspergillus nidulans* MAPK module AnSte11-Ste50-Ste7-Fus3 controls development and secondary metabolism. *Plos Genet.* **8**(7):470-471.
- Clarke, A., Desikan RHurst, R. D., Hancock, J. T. and Neill, S. J. 2000. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J.* **24**(5):667-677.
- Courtois, C., Besson, A., Dahan, J., Bourque, S., Dobrowolska, G., Pugin, A. and Wendehenne, D. 2008. Nitric oxide signaling in plants: interplays with Ca^{2+} and protein kinases. *J. Exp. Bot.* **59**(2):155-163.
- Du, H., Huang, Y. and Tang, Y. 2010. Genetic and metabolic engineering of isoflavonoid biosynthesis. *Appl. Microbiol. Biot.* **86**(5):1293-1312.
- Durner, J., Wendehenne, D. and Klessig, D. F. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *P. Natl. Acad. Sci. USA* **95**(17):10328-10333.
- Eilert, U. 1987. Elicitation: methodology and aspects of application. *Cell culture and somatic cell genetics of plants* **4**:153-196.
- Hahlbrock, K., Bednarek, P., Ciolkowski, I., Hamberger, B., Heise, A., Liedgens, H., Logemann, E., Nürnberger, T., Schmelzer, E. and Somssich, I. E. 2003. Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. *P. Natl. Acad. Sci. USA* **100**(suppl 2):14569-14576.

- Holley, S. R., Yalamanchili, R. D., Moura, D. S., Ryan, C. A. and Stratmann, J. W. 2003. Convergence of signaling pathways induced by systemin, oligosaccharide elicitors, and ultraviolet-B radiation at the level of mitogen-activated protein kinases in *Lycopersicon peruvianum* suspension-cultured cells. *Plant Physiol.* **132**(4):1728-1738.
- Jiao, C., Yang, R., Zhou, Y. and Gu, Z. 2016. Nitric oxide mediates isoflavone accumulation and the antioxidant system enhancement in soybean sprouts. *Food Chem.* **204**:373-380.
- Kumar, D. and Klessig, D. F. 2000. Differential induction of tobacco MAP kinases by the defense signals nitric oxide, salicylic acid, ethylene, and jasmonic acid. *Mol. Plant-Microbe In.* **13**(3):347-351.
- Masilamani, M., Wei, J. and Sampson, H. A. 2012. Regulation of the immune response by soybean isoflavones. *Immunol. Res.* **54**(1-3):95-110.
- Morris, P. C. 2001. MAP kinase signal transduction pathways in plants. *New Phytol.* **151**(1):67-89.
- Nakagami, H., Pitzschke, A. and Hirt, H. 2005. Emerging MAP kinase pathways in plant stress signalling. *Trend. Plant Sci.* **10**(7):339-346.
- Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D. and Hancock, J. T. 2002. Hydrogen peroxide and nitric oxide as signalling molecules in plants. *J. Exp. Bot.* **53**(372):1237-1247.
- Pagnussat, G. C., Lanteri, M. L., Lombardo, M. C. and Lamattina, L. 2004. Nitric oxide mediates the indole acetic acid induction activation of a mitogen-activated protein kinase cascade involved in adventitious root development. *Plant Physiol.* **135**(1):279-286.
- Pan, S., Borkovich, K. A., Pan, S. and Borkovich, K. A. 2008. A MAP kinase cascade required for regulation of development and secondary metabolism in *Neurospora crassa*. *Eukaryot. Cell* **7**(12):2113-2122.

- Pellinen, R. I., Korhonen, M.-S., Tauriainen, A. A., Palva, E. T. and Kangasjärvi, J. 2002. Hydrogen peroxide activates cell death and defense gene expression in birch. *Plant Physiol.* **130**(2):549-560.
- Ramani, S. and Chelliah, J. 2006. UV-B-induced signaling events leading to enhanced-production of catharanthine in *Catharanthus roseus* cell suspension cultures. *BMC Plant Biol.* **7**(1):61.
- Ramani, S., Patil, N. and Jayabaskaran, C. 2010. UV-B induced transcript accumulation of DAHP synthase in suspension-cultured *Catharanthus roseus* cells. *J. Mol. Signal.* **5**(1):1-9.
- Román, E., Arana, D. M., Alonso-Monge, R., Nombela, C. and Pla, J. 2007. MAP kinase pathways as regulators of fungal virulence. *Trends Microbiol.* **15**(4):181-190.
- Saito, H. 2010. Regulation of cross-talk in yeast MAPK signaling pathways. *Curr. Opin. Microbiol.* **13**(6):677-683.
- Singh, P., Ghosh, S. and Datta, A. 1997. A novel MAP-kinase kinase from *Candida albicans*. *Gene* **190**(1):99-104.
- Xiong, L. and Yang, Y. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *Plant Cell* **15**(3):745-759.
- Xu, M., Zhu, Y., Dong, J., Jin, H., Sun, L., Wang, Z., Lu, Z., Zhang, M. and Lu, D. 2012. Ozone induces flavonol production of *Ginkgo biloba* cells dependently on nitrate reductase-mediated nitric oxide signaling. *Environ. Exp. Botany* **75**:114-119.
- Yuasa, T., Ichimura, K., Mizoguchi, T. and Shinozaki, K. 2001. Oxidative stress activates ATMPK6, an Arabidopsis homologue of MAP kinase. *Plant Cell Physiol.* **42**(9):1012-1016.
- Zhang, A., Jiang, M., Zhang, J., Ding, H., Xu, S., Hu, X. and Tan, M. 2007. Nitric oxide induced by

hydrogen peroxide mediates abscisic acid-induced activation of the mitogen-activated protein kinase cascade involved in antioxidant defense in maize leaves. *New Phytol.* **175**(1):36-50.

For Review Only

Figure captions

Fig. 1. Induction of gene expression of *MAPK* by endogenous NO in soybean sprouts under UV-B radiation. Values are the mean and standard deviation of three replicates. Values not sharing the same letter are significantly different at $p < 0.05$.

Fig. 2. Induction of protein expression of *MAPK* by endogenous NO in soybean sprouts under UV-B radiation. Protein expression level of *MAPK* was determined on day 4. Panels show representative bands (A). Histograms represent relative protein level of MAPK (B) of 4-day-old soybean sprouts normalized to the corresponding rubisco. Values are the mean and standard deviation of three replicates. Values not sharing the same letter are significantly different at $p < 0.05$.

Fig. 3. Effects of NO-triggered MAPK on isoflavone content (A) and gene expression of CHS (B) and IFS (C) of soybean sprouts. Values are the mean and standard deviation of three replicates. Values not sharing the same letter are significantly different at $p < 0.05$.

Fig. 4. Effects of NO-triggered MAPK on protein expression of isoflavone biosynthetic-enzymes of soybean sprouts. Protein expression levels of CHS (B) and IFS (C) were determined on day 4. 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 and standard deviation of three replicates. Values not sharing the same letter are significantly different at $p < 0.05$.

Tables

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

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

Table 2. Proteins related to signal transduction in soybean sprouts that show significant changes after UV-B and SNP treatments.

Accession No.	Description	Fold Change	
		UV-B/CK	SNP/CK
gi 356534821	Mitogen-activated protein kinase kinase 2 isoform X3	1.975±0.183	2.028±0.142
gi 571505833	NAD(P)H-dependent 6'-deoxychalcone synthase isoform X1	2.164±0.086	2.052±0.239
gi 7288453	Isoflavone synthase 1, partial	1.909±0.041	1.874±0.093
gi 356531872	Calreticulin-3-like	1.693±0.284	2.046±0.155

Fold change > 1.5 represents up-regulation, while fold change < 0.67 represents down-regulation.

Fig. 1

Fig. 2

Fig. 3

Fig. 4