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BRIEF COMMUNICATION

Sodium nitroprusside modulates gene expression involved in glutathione synthesis in *Zea mays* leaves

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Abstract

To investigate a possible involvement of nitric oxide in gene regulation of glutathione and flavonoid synthesis pathways, maize seedlings were treated with sodium nitroprusside (SNP), a NO donor, and apocynin (APO), an inducer of NO production. After 12-h treatment, the transcripts of γ-glutamylcysteine synthetase (γ-*ecs*), glutathione synthetase (*gsh-s*), chalcone synthase (*chs*), phenylalanine ammonia lyase (*pal.1*), myb-related protein P (*P1*) and actin 1 (*act*) genes were quantified in maize leaves by real time PCR, using α-tubulin as standard transcript. The level of γ-*ecs* and *gsh-s* transcripts in maize leaves were increased 9-fold and 12-fold, respectively, following SNP treatment, while after APO treatment, those transcripts were not significantly different from control plants. SNP-treated maize leaves did not show significant changes in *pal.1* and *chs* expression. NO content in maize leaves was increased in SNP and APO treated plants in comparison to control plants. In conclusion, our experiments suggested that genes involved in glutathione synthesis could be modulated by SNP in maize leaves. On the other hand, APO had no effect on γ-*ecs* and *gshs* gene expression.

Additional key words: apocynin, glutamylcysteine synthetase, glutathione synthetase, maize, nitric oxide.

As a crucial gaseous signaling molecule in plants, nitric oxide (NO) plays significant roles in modulating several physiological and biochemical functions (Sakihama *et al.* 2003, Lei *et al.* 2007, López-Carrión *et al.* 2008, Wang *et al.* 2010). NO can directly interact with cellular constituents such as proteins and reactive oxygen species (ROS), although the ROS/NO interaction may be cytotoxic or protective depending on the relative ROS/NO concentrations (Beligni *et al*. 2002). NO may promote restoration of wounded tissue through stabilization of the cell redox state and stimulation of the wound scarring processes (Arasimowicz *et al.* 2009). NO could function as a signaling molecule in a cascade of events resulting in variation of gene expression (Lamattina *et al*. 2003, Pagnussat *et al*. 2002, Lamotte *et al*. 2004, Wilson *et al*. 2008). The S-nitrosylation of proteins by NO seems to be an especially important mechanism in the regulation of the function/activity of transcription factors (Grün *et al*. 2006, Serpa *et al*. 2007). Transcriptomic studies indicate that the genes regulated by NO sustain a large diversity of cellular functions, in accordance with the pleiotropic role of this molecule in plant physiology (Polverari *et al*. 2003, Parani *et al*. 2004). Changes in gene expression in response to 0.1 and

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Abbreviations: APO - apocynin; CHS - chalcone synthase; DAF-2DA - 4,5-diamino-fluorescein diacetate; GSH - reduced glutathione; GSHS - glutathione synthetase; GSNO - S-nitrosoglutathione; GSSG - oxidized glutathione; L-NAME - N^G-nitro-L-argininemethyl ester; NO - nitric oxide; NOC-18 - 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene; PAL - phenylalanine ammonia-lyase; ROS - reactive oxygen species; SNAP - S-nitroso-*N*-acetylpenicillamine; SNP - sodium nitroprusside; γ-ECS - γ-glutamylcysteine synthetase.

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1.0 mM sodium nitroprusside (SNP), a NO donor, were studied in *Arabidopsis* using the whole genome ATH1microarray and the authors observed 342 up-regulated and 80 down-regulated genes in response to NO treatment (Parani *et al*. 2004).

SNP is recognized as a NO donor (Floryszak-Wieczorek *et al*. 2006, Ederli *et al*. 2009), able to release this molecule when induced by light (Wang *et al*. 2002). Apocynin (4-hydroxy-3-methoxyacetophenone; aceto-vanillone, APO) is a methoxi-substituted cathecol able to raise the steady-state level of nitric oxide in maize seedling leaves (Tossi *et al*. 2009). The apocynin-induced NO production in maize leaves was greatly reduced by N^G-nitro-L-argininemethyl ester (L-NAME), a L-arginine-dependent NO synthase activity inhibitor, and 2-(4-carbo-xyphenyl)-4,4,5,5-tetra-methylimidazoline-1-oxyl-3-oxide, a specific NO scavenger (Tossi *et al*. 2009).

The thiol tripeptide glutathione (GSH; γ-glutamyl-cysteinylglycine) is the major low molecular mass thiol present in plant species. Glutathione synthesis takes place in two ATP-dependent steps, through reactions catalysed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSH-S), the rate-limiting enzyme being (γ-ECS) (Arisi *et al*. 1997, Noctor *et al*. 1998). The first reaction occurs in plastids and the second one in the cytosol (Galvez-Valdivieso and Mullineaux 2010). Analysis of the expression level of (γ-ECS) and GSH-S after treatment with sodium nitroprusside and S-nitroso-glutathione (GSNO), showed that *γ-ecs* and *gshs* genes are up regulated by NO treatment in *Medicago truncatula* roots (Innocenti *et al*. 2007). Changes in the GSH redox state as well as the regulation in ROS production could act as a signal stimulating plant growth (Díaz-Vivancos *et al*. 2010).

Flavonoids, derived from the phenylpropanoid skeleton, play important role in the plant-environment interactions (Lepiniec *et al*. 2006). Genes concerning flavonoid synthesis are regulated during plant development and in response to biotic and abiotic stresses (Schijlen *et al*. 2004). The biosynthesis of flavonoids is initiated by the enzymatic step catalysed by chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL) is the first enzyme of the overall phenylpropanoid biosynthetic pathway. SNP caused a rapid induction of both CHS and PAL transcripts in soybean cell extracts (Delledone *et al*. 1998). Furthermore, exogenous NO enhanced PAL activity in tobacco leaves (Durner *et al*. 1998) and in *Ginkgo biloba* callus (Hao *et al*. 2009). Pericarp color P (P1) is a myb-related transcriptional regulator involved in flavonoid biosynthesis in maize (Grotewold *et al*. 1998, Heine *et al*. 2007).

The aim of the present work was to evaluate the influence of sodium nitroprusside, a NO donor, and apocynin, a NO synthesis inducer, in expression of actin gene and genes involved in glutathione synthesis (*γ-ecs* and *gshs*) and phenylpropanoid pathway (*chs, pal* and *P1*).

Seeds of the maize (*Zea mays* L.) cv. SHS 3031 (kindly provided by *Santa Helena Sementes*, Sete Lagoas, MG, Brazil) were surface sterilized with 0.5 % hypochlorite for 20 min and 3 times washed with 70 % alcohol, and then rinsed with distilled water. The seeds were germinated on water-saturated filter paper at 25 °C in the dark. Germinated seedlings were transferred to a mixture of soil:*Vermiculite* (3:1 v/v) in a controlled growth chamber adjusted to 14-h photoperiod , irradiance of 150 μmol m^{-2} s^{-1}, temperature 25 °C and relative humidity 70 %, and weekly watered with modified Hoagland nutrient solution (Hoagland and Arnon 1950). After 14 d, maize seedlings were carefully excised at the base and placed in distilled water for 1 h. Then the seedlings were treated for 12 h with water (control treatment, H_2O), 100 μM apocynin (APO), 100 μM apocynin combined with 100 μM N^G-nitro-L-arginine-methyl ester (L-NAME), 100 μM sodium nitroprusside (SNP) or 100 μM potassium ferricyanide (FeCN). The third leaf of each plant was harvested and frozen in liquid nitrogen, and stored at -80 °C for further analysis. Three independent experiments were performed and two plants of each treatment were used for RNA isolation.

Approximately 75 mg of each plant was used for total RNA isolation using *RNAqueous®* kit (*Ambion*, Austin, TX, USA) and plant RNA isolation aid according to the manufacturer's protocol. RNA isolated was visualized by electrophoresis on agarose gels containing ethidium bromide, and its concentration was determined by a *Nanodrop ND 1000* spectrophotometer (*Thermo Fisher Scientific*, Wilmington, DE, USA). First strand cDNA was synthesized using 2 μg of isolated RNA, 0.5 μg oligo$(dT)_{18}$ primer, 0.5 μg random primer, 1 mM dNTP mix, 40 U RNasin ribonuclease inhibitor, 1× *AMV* reverse transcriptase reaction buffer and 30 U of *AMV* reverse transcriptase (*Promega*, Madison, WI, USA).

The *Primer Express™ v 3.0* software (*Applied Biosystems*, Foster City, CA, USA) was used to design the specific oligonucleotides used for real-time PCR (Table 1). Relative standard curves were constructed for each target gene compared with the constitutive gene (α-tubulin) in a serial 10-fold dilution starting from 10 ng. The reactions were performed using 0.005 cm^3 *SYBR Green I PCR Master Mix* 2 (*Applied Biosystems*), 300 nM of each primer and 0.002 cm^3 of template cDNA. All real time PCR (standards, samples and negative controls) were carried out in triplicates in adjacent wells on the same plate. Amplification reactions were run at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Immediately after each run, a dissociation curve was carried out at 95 °C for 1 min followed by 60 °C for 1 min. Real time PCR were performed on an *ABI PRISM™ 7500* detection system (*Applied Biosystems*). The experiment was conducted in a completely randomized design, in three lots. The data was analyzed by one-way *ANOVA* and compared by *F*-test, the means were compared using the Tukey's test. Data was analyzed using the *Statistica® v.7* (*StatSoft*, Tulsa, OK, USA) software package. Differences were considered to be significant when $P < 0.05$.

Table 1. Primers used in RT-PCR analysis. F - forward primer, R - reverse primer.

Gene		Primers	Fragment size	Accession No.
α-Tubulin	F	GCGCACCATCCAGTTCGT	60	X73980
	R	CTGGTAGTTGATTCCGCACTTG		
γ-Glutamylcysteine synthetase	F	TTCTTGGGCTTGGCTTTCA	62	AJ302783
	R	TTCCCTTTGGCATTATTGGTATGT		
Glutathione synthetase	F	GCTCCCAGCACATTTTCCA	99	AJ302784
	R	GAAATTCCCGTCCATGCTAACA		
Chalcone synthase	F	CGTCCGTCCGCAAATAATGT	99	CA851897
	R	ATGATGATTGTGCGACTGACAGT		
Myb-related protein P	F	AACGCTCCCACGACAATCA	101	NM_001111873
	R	GGCACGCACCTAAAGCAGAA		
Phenylalanine ammonia-lyase	F	CTCTTCGTTGTTGATTTTTGGTTGT	71	M95077
	R	AGAAAGAGCAACGCCACACA		
Actin	F	GCAGCATGAAGGTTAAAGTGATTG	61	NM_001155179.1
	R	GCCACCGATCCAGACACTGT		

NO content was measured in the second leaves after treatments for 12 h (SNP, APO, L-NAME, FeCN, H_2O). APO, L-NAME and H_2O plants were analyzed in one experiment and SNP and FeCN plants were analyzed in other experiment. The sections of leaves were placed in a solution of 5 μM 4,5-diaminofluorescein diacetate (DAF-2DA) for 30 min, in the dark under constant agitation. Then, the sections of leaves were washed with 2.5 mM HEPES buffer (pH 6.0) 3 times every 15 min to remove excess probe. The reaction of NO radical with DAF-FM DA results in the formation of the fluorescent triazole derivative DAF-2T (Modolo *et al.* 2006).The green fluorescence produced was visualized in *Olympus BX41* microscope (*Olympus*, Tokyo, Japan) and images acquired were analyzed using *QCapture PRO 5.1* (*QImaging*, Surrey, BC, Canada).

Maize seedlings were treated with sodium nitroprusside, a NO donor, and apocynin, an inducer of NO production. The transcripts of γ-glutamylcysteine synthetase *(γ-ecs)*, glutathione synthetase *(gsh-s)*, chalcone synthase *(chs)*, phenylalanine ammonia lyase (*pal.1)*, myb-related protein P (*P1)* and actin 1 (*act)* were quantified in maize leaves after 12-h treatment by real time PCR using relative standard curve with α-tubulin as standard transcript (Table 2). Changes in gene expression were estimated as the fold-change over the control plants, treated with water.

The level of γ-*ecs* transcripts in maize leaves increased 9.5-fold following SNP treatment. For the other treated leaves, γ-*ecs* transcript amounts were not significantly different in comparison to control plants. Concerning glutathione synthetase (*gsh-s*) expression, there was a 12.4-fold increase of transcript amounts for SNP treatment. For the other treatments, *gsh-s* amounts were not significantly changed in comparison to control plants (Table 2).

The *chs* transcript amount was 1.5-fold increased after SNP treatment, then decreased after APO treatment (0.25-fold), although no significant differences were observed in comparison to control plants. The observed *pal.1* transcript amount was unchanged for all treatments (Table 2).

Concerning the *P1* transcripts, increased (3-fold) amounts after SNP treatment and unchanged amount after other treatments were observed. The *act* transcript levels increased 2-fold after SNP treatment, and for the other treatments, *act* amounts were not significantly changed in comparison to control plants. After FeCN treatment, *act*

Table 2. Relative transcript amounts of *γ-ecs*, *gsh-s*, *P1*, *chs*, *pal.1* and *act* in maize leaves. Leaf samples of 14-d-old seedlings treated for 12 h with H_2O, apocynin (APO), apocynin plus N^G-nitro-L-argininemethyl ester (L-NAME), sodium nitroprusside (SNP) and ferricyanide (FeCN) were used for quantitative real time RT-PCR analysis. Relative amounts of transcripts were calculated using α-tubulin transcript as standard and the fold ratio is calculated with the corresponding water-treated sample. Data are presented as means ± SD, $n = 6$. Means followed by different letters in the column are significantly different at $P < 0.05$ according to Tukey test.

	γ-ecs	*gsh-s*	*P1*	*chs*	*pal.1*	*act*
H_2O	1.00 ± 0.38^{a}	1.00 ± 0.32^{a}	1.00 ± 0.74^{a}	1.00 ± 0.59^{ab}	1.00 ± 0.77^{a}	1.00 ± 0.32^{a}
APO	0.75 ± 0.48^{a}	3.54 ± 1.82^{a}	0.49 ± 0.48^{a}	0.25 ± 0.17^{a}	$0,77 \pm 0.54^{a}$	0.89 ± 0.50^{a}
L-NAME	0.73 ± 0.26^{a}	3.10 ± 0.91^{a}	0.36 ± 0.18^{a}	0.68 ± 0.25^{ab}	1.57 ± 0.66^{a}	0.63 ± 0.02^{a}
SNP	9.49 ± 2.28^{b}	12.44 ± 7.28^{b}	3.02 ± 1.42^{b}	1.47 ± 1.11^{b}	1.04 ± 0.93^{a}	2.07 ± 0.39^{b}
FeCN	0.64 ± 0.26^{a}	2.97 ± 1.24^{a}	0.61 ± 0.43^{a}	0.50 ± 0.30^{ab}	0.58 ± 0.50^{a}	1.31 ± 0.74^{ab}

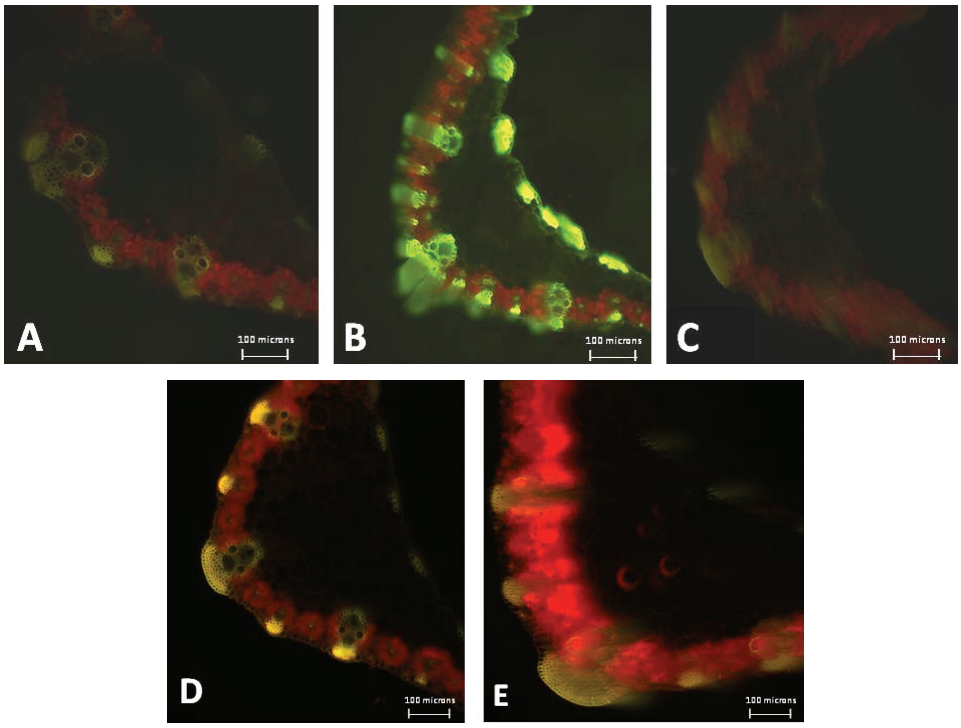

Fig. 1. Fluorescent micrographs indicating the NO presence in transversal sections of leaves (midrib) after 12-h H_2O (*A*), apocynin (*B*), apocynin plus N^G-nitro-L-argininemethyl ester (*C*), sodium nitroprusside (*D*) and ferricyanide (*E*) treatments.

transcript amount was not significantly different from that observed after SNP treatment (Table 2).

NO content was visualized as green fluorescence using the fluorophore DAF-2DA. After 12 h of treatment, green fluorescence signals were increased in apocynin treated leaves in comparison to water and L-NAME treated leaves (Fig. 1*A*,*B*,*C*), indicating that NO production was increased in apocynin treated leaves. The fluorescence emission was higher in SNP treated leaves than in FeCN treated (Fig. 1*D*,*E*), indicating increased NO production 12 h after SNP treatment.

It has been shown that apocynin was a strong inducer of NO production in leaves of maize seedlings, the maximum NO production being observed in response to 100 μM APO after 12-h treatment (Tossi *et al*. 2009). DAF-2DA fluorescence signal increased 12 h after apocynin treatment in our experiments, as previously reported (Tossi *et al*. 2009). These authors showed that APO induced the accumulation of NO in maize seedlings in a mechanism different from NADPH oxidase inhibition, conferring an augmented tolerance to oxidative stress produced by UV-B or H_2O_2. The use of apocynin was proposed as an alternative approach to study NO functionality in plants (Tossi *et al*. 2009): it could be an interesting way to discriminate from exogenous NO effects, once it is an inducer of endogenous NO.

In our experiments, maize leaves were treated with apocynin and with apocynin plus L-NAME. L-NAME is an inhibitor of L-arginine dependent NO synthase activity (Wilson *et al*. 2008). It was shown that the apocynin-induced NO production was greatly reduced by L-NAME in maize leaves (Tossi *et al*. 2009). In our experimental conditions, NO content, visualized as green fluorescence in the presence of DAF-2DA, was higher after 12-h APO treatment comparing to APO plus L-NAME and water treatments (Fig. 1*A*,*B*,*C*).

The most commonly used NO donor is SNP, an NO^+ donor (Ederli *et al*. 2009). The SNP solution is extremely photosensitive and its degradation is promoted by oxygen and temperature. Although the mechanism of NO release from SNP is not fully understood, it is clear that NO release requires either irradiation or a single-electron reduction (Wang *et al*. 2002). The measured half-life of SNP in solution was 12 h (Floryszak-Wieczorek *et al*. 2006). Exogenous SNP protected cucumber roots against oxidative stress induced by salt stress (Shi *et al*. 2007) and the function of NO in alleviation of oxidative stress was attributed to induction of various ROS-scavenging enzyme activities.

Maize leaves were treated with SNP, a NO donor, and also with the SNP degradation product FeCN, which is unable to release NO (Parani *et al*. 2004, Lei *et al.* 2007). The NO content was higher in the leaves after SNP treatment comparing to FeCN treatment (Fig. 1*D*,*E*).

After 12 h of treatment, SNP induced the expression of both transcripts related to glutathione synthesis, γ-*ecs* and *gshs*, of myb-related protein P transcript, *P1* and of actin transcript, *act* (Table 2). Meanwhile, SNP degradation product ferricyanide did not induce the expression of γ-*ecs, gsh-s* and *P1*. Therefore the observed increase in transcript levels of γ-*ecs*, *gsh-s* and *P1* after 12 h of SNP

treatment was attributable to NO released. In maize leaves, glutathione synthesis related enzymes, γ-ECS and GSH-S transcripts and proteins were found in both bundle sheath and mesophyll cells under optimal conditions (Gomez *et al*. 2004).

In our experiments, γ-*ecs* and *gshs* transcripts were significantly increased after SNP treatment. Similarly to our results, the transcript levels of γ-*ecs* and *gshs* increased 3-fold and 8-fold, respectively, in SNP-treated roots of *Medicago truncatula* (Innocenti *et al*. 2007). Our experiments demonstrated that glutathione synthesis could be modulated by SNP in maize leaves. Glutathione biosynthesis is associated with altered expression of some stress-responsive genes prior to or during the exposure to biotic and abiotic stress (Galvez-Valdivieso and Mullineaux 2010). Glutathione is a key arbiter of the intracellular redox potential. Protein glutathionylation can modulate enzyme activity by modification of catalytic site Cys residues or affect biological activity by competing with other thiol modifications (Foyer and Noctor 2005).

NO can react with GSH to form GSNO. This molecule can then function in trans-nitrosylation reactions passing on its NO group to reactive Cys thiols within target proteins. Therefore, GSNO can function as a source of NO to support protein S-nitrosylation. As GSNO is a relatively stable molecule, it might also operate in NO storage and transfer (Spadaro *et al*. 2010). Intracellular NO removal can involve the NO reaction with GSH to form GSNO and the subsequent reduction of GSNO to GSSG and nitrate (Baudouin 2011).

Otherwise, SNP-treated maize leaves did not show significant changes in *pal.1* and *chs* expression (Table 2). Differently from our results, *pal* expression was induced in NO-treated tobacco leaves (Durner *et al*. 1998) and *pal* and *chs* transcripts were increased in SNP-treated soybean (Delledonne *et al*. 1998). The PAL activity of pelargonium leaves was activated by SNP under irradiance, not in the dark. Pelargonium leaf discs treated with 100 μM SNP under irradiance showed a stimulation of PAL activity from 1 to 24 h after the beginning of treatment (Floryszak-Wieczorek *et al*. 2006).

In plants, large-scale gene expression analyses have identified many genes whose expression is also regulated by NO. The conclusion, from these studies is that NO-responsive genes are, for the most part, stress-related and serve a variety of functions ranging from plant defense, oxidative stress responses and hormone interplay to metabolism and development (Moreau *et al*. 2010). In conclusion our results showed that NO content increased in maize leaves after 100 μM apocynin or 100 μM SNP treatment, and γ-*ecs* and *gsh-s* genes involved in glutathione synthesis could be modulated by SNP treatment. On the other hand, apocynin treatment had no effect on γ-*ecs* and *gsh-s* gene expression in maize leaves.

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