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The regulation of transcription of genes related to oxidative stress and glutathione synthesis in *Zea mays* leaves by nitric oxide

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Abstract

Apocynin was used as an inducer of nitric oxide (NO) synthesis in leaves of two maize (*Zea mays* L.) cultivars (SHS 3031 and DKB 240). The transcription of γ-glutamylcysteine synthetase *(γ-ecs*), glutathione synthetase (*gshs*), chalcone synthase (*chs*), flavanone 3-β-hydroxylase (*fht1*), glutathione S-transferase (*gst23*), ascorbate peroxidase (*apx1, apx2*), catalase (*cat1, cat3*), and superoxide dismutase (*sod2*) genes were quantified in leaves of maize and NO presence was detected by fluorescence microscopy. An increase in the fluorescence after apocynin treatment was not observed in leaves of DKB 240 but leaves of SHS 3031 showed higher fluorescence after 6 and 12 h of apocynin treatment. We observed that *γ-ecs*, *apx1, cat1*, and *sod2* transcription increased after 24 h of apocynin treatment in SHS 3031.

Additional key words: antioxidants, apocynin, gene expression, maize, qRT-PCR.

Introduction

Nitric oxide acts as a key signaling molecule in many plant processes including responses to drought and salinity (Zeng *et al*. 2011). Large-scale gene expression analysis has identified many NO-responsive genes that are related to stress, plant defense, and hormonal responses (Moreau *et al.* 2010). NO-induced long term UV-B protection in maize requires a functional *ZmP* gene. *ZmP* encodes a MYB-like transcriptional factor that activates chalcone synthase (*chs*) and chalcone isomerase (*chi*) genes involved in the flavonoid biosynthesis. In maize leaves, *ZmP*, *chs,* and *chi* expressions are up-regulated by UV-B in a NO-dependent manner (Tossi *et al.* 2011).

Exogenous NO stimulates glutathione (GSH) synthesis in plants (Innocenti *et al.* 2007, Mello *et al.* 2012) that occurs in two ATP-dependent steps catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSHS). It was shown that *γ-ecs* and *gshs* genes are up-regulated by NO treatment in *Medicago truncatula* (Innocenti *et al.* 2007, Meilhoc *et al.* 2011). Many GSH functions are linked to reversible redox reactions of the cysteine sulfur group (Noctor *et al*. 2002, Foyer and Noctor 2005). GSH conjugates with a vast range of endogenous compounds and xenobiotics *via* glutathione S-transferase (GST) rendering them less reactive and more water-soluble and thus more tractable for vacuolar sequestration (Wisser *et al.* 2011) or transport. GSH interacts with NO forming S-nitroso-glutathione, a potential signaling molecule or a reservoir of NO (Baudouin 2011).

Apocynin is used as an inducer of NO synthesis in maize leaves (Tossi *et al*. 2009a). To investigate whether the NO induced by apocynin can interfere with glutathione metabolism, the transcription of *γ-ecs*, *gshs,* and *gst23* (encoding a type III GST; McGonigle *et al.*

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Abbreviations: APO - apocynin; *apx* - gene encoding ascorbate peroxidase; *cat* - gene encoding catalase; *chi* - gene encoding chalcone isomerase; *chs* - gene encoding chalcone synthase; cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA - 4,5-diaminofluorescein diacetate; *fht* - gene encoding flavanone 3-β-hydroxylase; *γ-ecs* - gene encoding γ-glutamylcysteine synthetase; GSH - glutathione; *gshs* - gene encoding glutathione synthetase; *gst* - gene encoding glutathione S-transferase; qRT-PCR - quantitative reverse transcription polymerase chain reaction; ROS - reactive oxygen species; SNP - sodium nitroprusside; *sod* - gene encoding superoxide dismutase.

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2000) were analyzed in leaves of maize cvs. DKB 240 and SHS 3031. We also analyzed transcript amounts of *chs* and flavanone 3-β-hydroxylase (*fht1*), both involved in flavonoid synthesis (Deboo *et al.* 1995, Lillo *et al.* 2008). To investigate the effects of NO-induced by apocynin on gene expression related to oxidative stress response, ascorbate peroxidase (*apx1, apx2*), catalase (*cat1, cat3*), and superoxide dismutase (*sod2*) transcript levels were analyzed in leaves of SHS 3031 in this work.

Materials and methods

Maize (*Zea mays* L. cvs. DKB 240 and SHS 3031) seeds were germinated on water-saturated paper at 25 °C in a dark chamber for 3 d. Germinated seedlings were transferred to soil and kept in a growth chamber for 7 d (a 14-h photoperiod, irradiance of 150 μmol m^{-2} s^{-1}, temperature of 25 °C, and relative humidity of 70 %) and watered daily. The seedlings were carefully excised at the base of stem and placed in distilled water for 1 h. Then, the seedlings were transferred to water (control treatment, H_2O), 100 μM apocynin (4-hydroxy-3-methoxyacetophenone, APO), or 100 μM apocynin combined with 100 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). After 0, 2, 6, 12, and 24 h, the second leaves were sampled, immediately frozen in liquid nitrogen, and stored at -80 °C for further analyses. Three independent experiments (biological replicates) were performed.

NO presence was detected according to Tossi (2009a). The leaf sections were placed in a solution of 5 μM 4,5-diaminofluorescein diacetate (DAF-FM DA) (*Sigma*, St. Louis, USA) in the dark under constant agitation for 30 min. Then, the sections were washed in 2.5 mM HEPES buffer (pH 6.0) 3 times, each washing lasting 15 min, to remove the probe excess. The green fluorescence was visualized in *Olympus BX41* microscope (*Olympus*, Tokyo, Japan) and acquired images were analyzed using *QCapture PRO 5.1* (*QImaging*, Surrey, Canada). The samples of zero time (1-h treatment in distilled water) were used as controls showing the basal levels of NO.

For the RNA isolation, three plants of each treatment and of each biological replicate were used, so nine isolated RNAs were prepared for each condition. The total RNA was isolated from approximately 100 mg of leaf tissue using *RNeasy*® mini kit (*Qiagen*, Chatsworth, CA, USA) according to the instructions supplied by the manufacturer. RNA samples were treated with DNase I amplification grade (*Sigma*) to eliminate DNA contamination. RNA was quantified by the *Nanodrop 2000* spectrophotometer (*Thermo Fisher Scientific*, Wilmington, DE, USA). After quantification, reverse transcription was performed using 2 μg of RNA oligo(dT) primer and high capacity cDNA archive kit (*Life Technologies,* Carlsbad, CA, USA) following the manufacturer's instructions.

The *Primer Express™ v. 3.0* software (*Applied Biosystems*, Foster City, CA, USA) was used to design the specific oligonucleotides for real-time PCR (Table 1). To determine amplification efficiencies, cDNA of a control sample (water treatment) was serially diluted with water (5-fold dilution from 100 ng to 0.16 ng of cDNA template) and relative standard curves of each target gene were compared to the constitutive gene (*α-tubulin*) in triplicate. Amplification efficiencies were determined using the following equation: efficiency = $10^{(-1/\text{slope})}$ - 1, where the slope is the value obtained from the standard curve. The sample reactions were performed using *SYBR Green PCR Master Mix 2X* (*Life Technologies*) and 40 ng of template cDNA. Amplification reactions of samples were carried out in duplicate in adjacent wells on the same plate, 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, with linear increase from 60 to 95 °C, performed on an *ABI PRISM*™ *7500* detection system (*Life Technologies*). The transcript quantifications of *γ-ecs, gshs, chs, act, fht1, gst23, apx1, apx2*, *cat1, cat3*, and *sod2* were performed in relation to *α-tubulin* (a reference gene) and they were calculated by $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). Changes in transcript amounts were analyzed by non-parametric Kruskal-Wallis test and compared by Student *t*-test ($P < 0.05$ for significant differences). Statistical analysis was performed using the *R software v. 2.12.1* (http://www.R-project.org/).

Results

Leaves of maize cvs. SHS 3031 and DKB 240 were treated with water, NO inducer apocynin, or apocynin plus cPTIO, a NO scavenger. After 2 h of the apocynin treatment, differences in fluorescence were not observed in both cvs. After 6 and 12 h of the apocynin treatment, higher fluorescence was observed in leaves of SHS 3031 but not in DKB 240 compared to water treated leaves. The SHS 3031 samples treated with apocynin combined with cPTIO presented lower fluorescence than those treated only with apocynin (Figs. 1, 2).

The transcription of *γ-ecs*, *gshs*, *chs*, *fht1*, and *gst23* after the apocynin, apocynin plus cPTIO, and water treatments were analyzed in leaves of DKB 240 and SHS 3031 whereas the transcription of two ascorbate peroxidase genes (*apx1, apx2*), two catalase genes (*cat1, cat3*), and one superoxide dismutase gene (*sod2*) were analyzed only in leaves of SHS 3031. The amplification efficiencies were in the range of 90 - 110 % (Livak and

Table 1. Primer forward (F) and reverse (R) sequences and concentrations used in qRT-PCR analysis for *Zea mays* transcript quantification.

Genes		Primers	Concentration [nM]	Fragment length [bp]	Accession No.
α-tubulin	F	GCGCACCATCCAGTTCGT	300	60	X73980
	R	CTGGTAGTTGATTCCGCACTTG	300		
γ-glutamylcysteine synthetase	F	TTCTTGGGCTTGGCTTTCA	300	62	AJ302783
	R	TCCCTTTGGCATTATTGGTATGT	300		
glutathione synthetase	F	GCTCCCAGCACATTTTCCA	300	99	AJ302784
	R	GAAATTCCCGTCCATGCTAACA	300		
chalcone synthase	F	CGTCCGTCCGCAAATAATGT	300	99	CA851897
	R	ATGATGATTGTGCGACTGACAGT	300		
actin1	F	GCAGCATGAAGGTTAAAGTGATTG	300	61	NM_001155179.1
	R	GCCACCGATCCAGACACTGT	300		
flavanone 3-β-hydroxylase	F	CAGCAGAGTGCCAACAAGGA	500	72	U04434.1
	R	CCCTCTACCATATCAGGCAAAAA	500		
glutathione S-transferase 23	F	GGCTAGTAATTCTGGAGCAGCTAGTT	500	76	NM_001111524.1
	R	GCAAAAGTGCAACCAGTCCTTA	500		
ascorbate peroxidase 1	F	CACCTCAAGCTCTCCGAACTG	500	63	NM_001156720.1
	R	GTCCCGCTCCAGGATATGG	100		
ascorbate peroxidase 2	F	AGGCCTTCTGCAGCTACCAA	500	58	NM_001112030.1
	R	TGGGCGGAAGGATGGAT	100		
catalase 1	F	GATGGGTTGACGCACTGACA	500	60	NM_001111945.1
	R	AGATCCAAATGGTACGGTGTTCA	300		
catalase3	F	TCGCTCGGACACCCAAAG	500	59	NM_001111946.1
	R	GGCGAGGAGGTCTATCCAGAT	500		
superoxide dismutase 2	F	CAGAGACAATGGTGAAGGCAGTT	500	61	M54936.1
	R	TGATGTCAAGGGCACCATCTT	100		

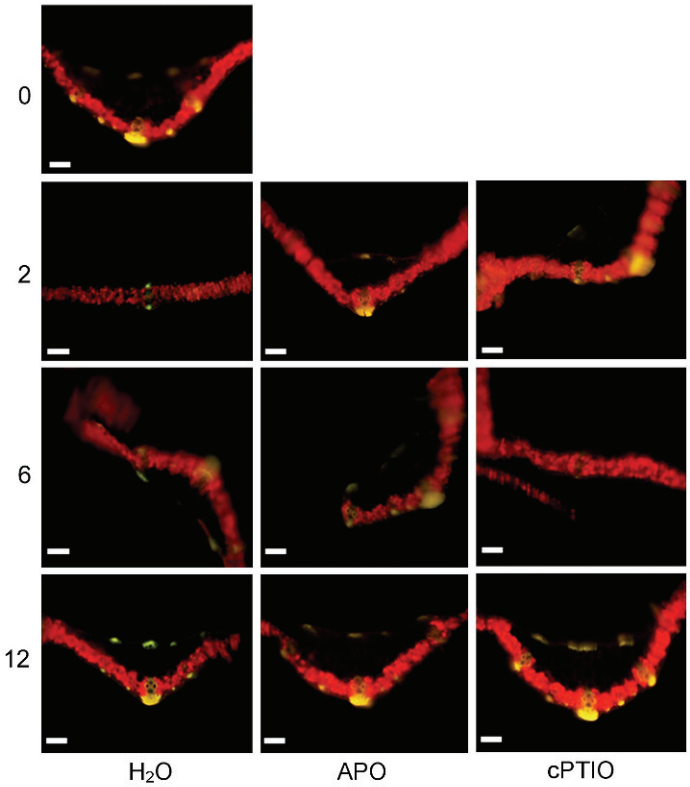

Fig. 1. NO visualization by fluorescence microscopy in transversal sections of *Zea mays* DKB 240 leaves (midrib) loaded with DAF-FM DA. Leaf samples treated for 0, 2, 6, and 12 h with water (H_2O), apocynin (APO), APO plus cPTIO. *Scale bar* 100 µm.

Schmittgen 2001). Regarding DKB 240, increases in *γ-ecs* transcript amounts were observed after 24 h of the apocynin treatment and addition of cPTIO did not abolish this observed increase (Table 2). For SHS 3031, 2-fold and 2.6-fold increases in *γ-ecs* and *act* transcript amounts were observed, respectively, 24 h after the apocynin treatment compared to the water treatment, but these increases were suppressed by cPTIO presence (Table 3). Regarding oxidative stress response genes in SHS 3031, *apx1* decreased 0.6-fold after 12 h of the apocynin treatment whereas 2-fold increase in *apx1, cat1*, and *sod2* transcription was observed 24 h after the apocynin treatment, and these changes were also suppressed by cPTIO (Table 4). Regarding *chs, fht1*, and *gst23*, transcript amounts were not statistically different after apocynin treatment for both maize cultivars (Tables 2 and 3).

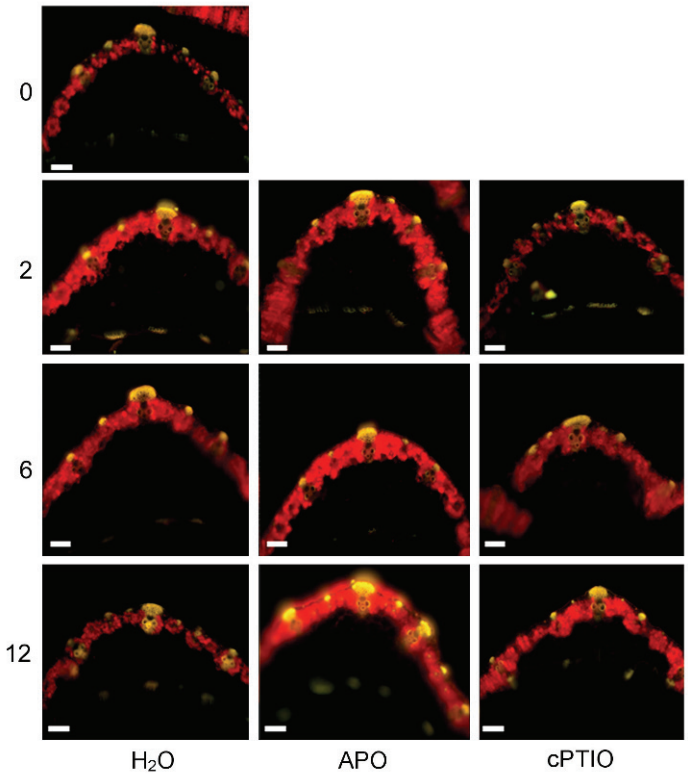

Fig. 2. NO visualization by fluorescence microscopy in transversal sections of *Zea mays* SHS 3031 leaves (midrib) loaded with DAF-FM DA. Leaf samples treated for 0, 2, 6, and 12 h with water (H_2O), apocynin (APO), APO plus cPTIO. *Scale bar* 100 µm.

Discussion

Apocynin was used in a study of the NO functionality in plants. It is methoxy-substituted cathecol able to raise the steady-state level of NO in maize, and this compound, a NADPH oxidase inhibitor, induces the accumulation of endogenous NO through a nitric oxide synthase (NOS)-like activity (Tossi *et al*. 2009a,b). Thus, an increase in the NO fluorescence due to the reaction between DAF-FM DA and NO is expected in the samples treated with apocynin. On the other hand, the NO scavenger cPTIO should cause a decrease in the fluorescence in the samples treated with apocynin plus cPTIO. The NO detection by microscopy through the diaminofluorescein reaction was commonly used to visualize NO in many previous studies (Creus *et al*. 2005, Arasimowicz-Jelonek *et al.* 2009, Lombardi *et al*. 2010, Corpas *et al*. 2011, Tossi *et al.* 2011).

In a previous study with leaves of maize cv. SHS 3031, we applied apocynin as NO inducer and sodium nitroprusside (SNP) as NO donor, and an increase in fluorescence was observed after 12 h of NO treatments (Mello *et al.* 2012). A time-course experiment of endogenous NO production in maize cv. N107B leaves treated with 100 µM apocynin, the same concentration as used in our study, shows increased fluorescence already after 2 h of the treatment, however, after 24 h of the treatment, NO diffuses over the whole leaf (Tossi *et al.* 2009a). In our work, it is shown that apocynin is able to induce the endogenous NO production after 6-h treatment reaching the maximum fluorescence after 12 h in the leaves of cv. SHS 3031 (Fig. 2) whereas we did not observe any fluorescence increase in the cv. DKB 240 (Fig. 1). These differences could be related to different adaptive responses to environmental stresses between these two maize cultivars.

Table 2. Relative expression of *γ-ecs, gshs, chs, act, fht1*, and *gst23* in *Zea mays* DKB 240 leaves. Leaf samples of 10-d-old seedlings were treated for 2, 6, 12, and 24 h with H_2O, APO, and APO plus cPTIO. Relative content of transcripts were calculated by qRT-PCR using the $\Delta\Delta C_T$ method and *α*-tubulin as a reference gene. Means ± SD, $n = 9$. Values followed by different letters in the column are significantly different at $P < 0.05$ according to the Kruskal-Wallis test.

Time [h]	Treatment	γ-*ecs*	*gshs*	*chs*	*act*	*fht1*	*gst23*
2	H_2O	1.15 ± 0.60^{a}	1.20 ± 0.82^{a}	1.13 ± 0.50^{a}	1.11 ± 0.59^{a}	2.91 ± 4.38^{a}	1.03 ± 0.27^{a}
	APO	3.20 ± 3.47^{a}	1.44 ± 1.26^{a}	6.03 ± 10.79^{a}	1.00 ± 0.48^{a}	5.31 ± 7.53^{a}	3.66 ± 6.08^{a}
	cPTIO	5.42 ± 7.50^{a}	1.74 ± 1.84^{a}	7.04 ± 11.36^{a}	2.54 ± 3.97^{a}	9.12 ± 9.45^{a}	4.40 ± 7.95^{a}
6	H_2O	1.14 ± 0.57^{a}	1.07 ± 0.38^{a}	1.13 ± 0.52^{a}	1.14 ± 0.6^{a}	1.51 ± 1.65^{a}	2.07 ± 2.76^{a}
	APO	0.81 ± 0.27^{ab}	1.13 ± 0.41^{a}	1.69 ± 0.90^{a}	0.97 ± 0.77^{a}	2.55 ± 2.20^{a}	0.75 ± 0.83^{a}
	cPTIO	0.59 ± 0.34^{b}	1.04 ± 0.97^{a}	0.98 ± 0.67^{a}	1.21 ± 1.47^{a}	1.88 ± 2.32^{a}	2.26 ± 4.46^{a}
12	H_2O	1.15 ± 0.63^{a}	1.35 ± 1.09^{a}	1.12 ± 0.49^{a}	0.84 ± 0.46^{a}	0.88 ± 0.34^{a}	1.81 ± 1.71^{a}
	APO	0.94 ± 0.60^{a}	1.81 ± 1.25^{a}	1.32 ± 0.93^{a}	0.81 ± 0.81^{a}	1.04 ± 0.53^{a}	0.89 ± 0.90^{a}
	cPTIO	0.87 ± 0.44^{a}	1.34 ± 0.66^{a}	1.09 ± 0.46^{a}	0.71 ± 0.53^{a}	1.17 ± 0.92^{a}	0.74 ± 0.54^{a}
24	H_2O	1.08 ± 0.52^{b}	1.08 ± 0.41^{a}	1.29 ± 1.18^{a}	0.96 ± 0.33^{a}	1.55 ± 2.03^{a}	1.59 ± 1.99^{a}
	APO	1.74 ± 1.14^{a}	1.44 ± 0.92^{a}	1.26 ± 0.68^{a}	1.40 ± 1.10^{a}	1.30 ± 1.11^{a}	1.16 ± 1.07^{a}
	cPTIO	1.36 ± 0.55^{ab}	0.64 ± 0.46^{b}	1.24 ± 0.87^{a}	1.07 ± 0.38^{a}	0.61 ± 0.21^{a}	0.98 ± 0.94^{a}

Table 3. Relative expression of *γ-ecs, gshs, chs, act, fht1*, and *gst23* in *Zea mays* SHS 3031 leaves. Leaf samples of 10-d-old seedlings were treated for 2, 6, 12, and 24 h with H_2O, APO, and APO plus cPTIO. Relative content of transcripts were calculated by qRT-PCR using the $\Delta\Delta C_T$ method and *α*-tubulin as a reference gene. Means ± SD, $n = 9$. Values followed by different letters in the column are significantly different at $P < 0.05$ according to the Kruskal-Wallis test.

Time [h]	Treatment	γ-*ecs*	*gshs*	*chs*	*act*	*fht1*	*gst23*
2	H_2O	0.92 ± 0.50^{a}	1.21 ± 1.00^{a}	1.38 ± 1.18^{a}	1.16 ± 0.68^{a}	2.28 ± 2.41^{a}	1.36 ± 0.93^{a}
	APO	0.89 ± 0.52^{a}	1.17 ± 0.75^{a}	2.06 ± 2.07^{a}	1.38 ± 0.53^{a}	1.12 ± 1.49^{a}	1.25 ± 1.01^{a}
	cPTIO	0.85 ± 0.24^{a}	1.27 ± 0.59^{a}	2.51 ± 3.33^{a}	2.87 ± 2.69^{a}	4.81 ± 6.35^{a}	4.15 ± 5.06^{a}
6	H_2O	1.05 ± 0.34^{b}	1.03 ± 0.25^{a}	1.27 ± 0.90^{a}	1.04 ± 0.34^{a}	2.70 ± 4.18^{a}	1.44 ± 1.33^{a}
	APO	3.40 ± 2.44^{a}	2.38 ± 2.12^{a}	3.85 ± 3.57^{a}	1.38 ± 0.73^{a}	16.58 ± 30.09^{a}	1.53 ± 1.24^{a}
	cPTIO	$2.11 \pm .1.50^{a}$	3.70 ± 7.71^{a}	1.31 ± 1.03^{a}	1.56 ± 1.00^{a}	3.79 ± 6.90^{a}	4.23 ± 6.95^{a}
12	H_2O	1.27 ± 0.87^{a}	1.21 ± 0.77^{a}	2.25 ± 3.05^{a}	1.18 ± 0.76^{a}	4.40 ± 6.66^{a}	1.73 ± 1.76^{a}
	APO	1.37 ± 1.04^{a}	1.79 ± 1.54^{a}	0.63 ± 0.41^{a}	0.81 ± 0.46^{a}	0.65 ± 0.70^{a}	1.25 ± 1.17^{a}
	cPTIO	1.50 ± 1.08^{a}	3.16 ± 4.96^{a}	1.15 ± 0.92^{a}	1.44 ± 0.86^{a}	14.48 ± 19.59^{a}	3.92 ± 4.08^{a}
24	H_2O	1.12 ± 0.57^{b}	1.12 ± 0.61^{a}	1.69 ± 1.84^{a}	1.16 ± 0.76^{b}	2.62 ± 3.71^{a}	1.35 ± 1.30^{a}
	APO	2.11 ± 0.91^{a}	1.45 ± 0.48^{a}	2.59 ± 4.31^{a}	2.59 ± 1.79^{a}	20.63 ± 36.79^{a}	4.35 ± 6.27^{a}
	cPTIO	1.23 ± 0.87^{b}	1.20 ± 0.58^{a}	0.82 ± 0.77^{a}	0.99 ± 0.56^{b}	2.39 ± 2.91^{a}	4.15 ± 5.40^{a}

Moreover, as a control for NO detection, the SHS 3031 leaves were treated with apocynin plus cPTIO and these samples presented lower fluorescence compared to samples treated only with apocynin. These results are in agreement with previous data showing cPTIO as an efficient NO scavenger (Modolo *et al*. 2006, Ahlfors *et al*. 2009, Tossi *et al*. 2009b, De Osti *et al*. 2010, Kovacik *et al*. 2010).

There is evidence that NO regulates gene expressions (Lombardi *et al.* 2010). NO activates signaling pathways resulting in changes in gene expressions through intermediate cellular messengers (Tossi *et al.* 2011). Furthermore, nuclear regulatory proteins are affected by NO, since the S-nitrosylation of proteins is an important mechanism in regulation of the function of transcription factors (Lindermayr *et al.* 2005, Serpa *et al.* 2007).

Regarding the glutathione synthesis related genes, *γ-ecs* transcription was increased after 24 h of apocynin treatment (Table 3). The stimulation of GSH synthesis by NO was shown in *Medicago truncatula*, where the amounts of *γ-ecs* and *gshs* transcripts increase following SNP treatment (Innocenti *et al.* 2007). The NO accumulation increases γ-ECS activity and the total glutathione content in *Agropyron cristatum* leaves under water stress (Shan *et al.* 2012). In our previous study with *Zea mays* SHS 3031 leaves, we showed that *γ-ecs* transcript amount was increased 12 h after SNP treatment. In this previous work, we measured *γ-ecs* transcript amounts only 12 h after apocynin treatment (Mello *et al.* 2012), and it was not changed compared to water treatment. Similar results were obtained in this work (Table 3). We observed that the exogenous NO produced by SNP (Mello *et al.* 2012) and endogenous NO induced by apocynin induced *γ-ecs* expression in different time courses, after 12 h and 6 h,

Table 4. Relative expression of *apx1, apx2, cat1, cat3*, and *sod2* in *Zea mays* SHS 3031 leaves. Leaf samples of 10-d-old seedlings were treated for 2, 6, 12, and 24 h with H_2O, APO, APO plus cPTIO. Relative content of transcripts were calculated by qRT-PCR using the $\Delta\Delta C_T$ method and α-tubulin as a reference gene. Means ± SD, $n = 9$. Values followed by different letters in the column are significantly different at $P < 0.05$ according to the Kruskal-Wallis test.

Time [h]	Treatment	*apx1*	*apx2*	*cat1*	*cat3*	*sod2*
2	H_2O	1.14 ± 0.66^{ab}	1.39 ± 1.31^{a}	1.14 ± 0.65^{a}	1.58 ± 1.33^{a}	1.09 ± 0.54^{a}
	APO	0.86 ± 0.37^{b}	1.43 ± 1.14^{a}	1.11 ± 0.48^{a}	3.22 ± 2.87^{a}	0.96 ± 0.52^{a}
	cPTIO	2.03 ± 1.67^{a}	2.15 ± 1.98^{a}	3.00 ± 5.24^{a}	3.50 ± 3.13^{a}	1.69 ± 1.29^{a}
6	H_2O	1.06 ± 0.36^{a}	1.03 ± 0.25^{a}	1.12 ± 0.58^{a}	1.31 ± 1.04^{a}	1.06 ± 0.36^{a}
	APO	0.87 ± 0.37^{a}	1.30 ± 0.77^{a}	1.67 ± 1.00^{a}	2.05 ± 1.54^{a}	2.18 ± 1.52^{a}
	cPTIO	0.99 ± 0.65^{a}	1.92 ± 2.27^{a}	2.50 ± 2.41^{a}	$2,69 \pm 1.97^{a}$	2.21 ± 2.10^{a}
12	H_2O	1.03 ± 0.25^{a}	1.04 ± 0.32^{ab}	1.02 ± 0.21^{a}	1.23 ± 0.78^{a}	1.07 ± 0.40^{a}
	APO	0.66 ± 0.35^{b}	0.87 ± 0.34^{b}	0.82 ± 0.41^{a}	2.49 ± 4.15^{a}	1.21 ± 0.65^{a}
	cPTIO	1.27 ± 0.59^{a}	1.46 ± 0.78^{a}	1.15 ± 0.76^{a}	2.29 ± 1.45^{a}	1.71 ± 0.79^{a}
24	H_2O	1.05 ± 0.38^{b}	1.07 ± 0.36^{a}	1.11 ± 0.57^{b}	1.04 ± 0.45^{a}	1.05 ± 0.38^{b}
	APO	2.03 ± 1.15^{a}	1.79 ± 1.04^{a}	1.82 ± 1.18^{a}	1.21 ± 0.83^{a}	1.88 ± 0.97^{a}
	cPTIO	1.56 ± 0.69^{b}	0.91 ± 0.59^{a}	0.93 ± 0.81^{b}	0.93 ± 0.60^{a}	1.21 ± 1.17^{b}

respectively. SNP and apocynin treatments caused changes of *γ-ecs* expression in maize leaves.

Concerning the genes coding oxidative stress response enzymes, transcription of *apx1, cat1*, and *sod2* increased (2-fold) after 24 h of apocynin treatment in the SHS 3031 leaves (Table 4) which is in agreement with previous work showing increase in the transcript amounts and the total activities of antioxidant enzymes CAT and APX induced by SNP, and these changes were substantially arrested by the pre-treatment with cPTIO (Zhang *et al.* 2007). Our results strengthen the induction of antioxidant enzyme expressions, once a NO inducer has been used instead of a NO donor. It is necessary to confirm the observed changes by using different NO modulator compounds (Arasimowicz-Jelonek *et al.* 2011).

Several putative NO-responsive genes have been identified in plant tissues and cell suspensions with altered transcription. A significant part of these genes encode proteins related to plant adaptive responses to biotic and abiotic stresses (Besson-Bard *et al.* 2009). In leaves of the apocynin-responsive maize cv. SHS 3031, NO presence was revealed by fluorescence microscopy and we observed increases of transcription of *γ-ecs, apx1, cat1*, and *sod2* 24 h after the treatment. Our data confirm the emerging picture that NO acts as a signal involved in the plant adaptive response to environmental stresses (Besson-Bard *et al.* 2009) although differences in transcript amounts of other analyzed genes (*chs, fht1* and *gst23*) were not observed after the apocynin treatment of the maize leaves.

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