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Nitric oxide is required for determining root architecture and lignin composition in sunflower. Supporting evidence from microarray analyses

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ABSTRACT

Nitric oxide (NO) is a signal molecule involved in several physiological processes in plants, including root development. Despite the importance of NO as a root growth regulator, the knowledge about the genes and metabolic pathways modulated by NO in this process is still limited. A constraint to unravel these pathways has been the use of exogenous applications of NO donors that may produce toxic effects. We have analyzed the role of NO in root architecture through the depletion of endogenous NO using the scavenger cPTIO. Sunflower seedlings growing in liquid medium supplemented with cPTIO showed unaltered primary root length while the number of lateral roots was deeply reduced; indicating that endogenous NO participates in determining root branching in sunflower. The transcriptional changes induced by NO depletion have been analyzed using a large-scale approach. A microarray analysis showed 330 genes regulated in the roots ($p \leqslant 0.001$) upon endogenous NO depletion. A general cPTIO-induced up-regulation of genes involved in the lignin biosynthetic pathway was observed. Even if no detectable changes in total lignin content could be detected, cell walls analyses revealed that the ratio G/S lignin increased in roots treated with cPTIO. This means that endogenous NO may control lignin composition *in planta*. Our results suggest that a fine tuning regulation of NO levels could be used by plants to regulate root architecture and lignin composition. The functional implications of these findings are discussed.

1. Introduction

Root architecture is a relevant component of the plant adaptive capacity to its environment since it provides the plant with a secure supply of nutrients and water, as well as anchorage and support. The root system is composed of a primary root (PR) and a bulk of lateral roots (LR). While PR growth is established during embryogenesis and is regulated through the activity of the apical meristem, LR number and length is not predetermined and results from a complex integration of endogenous and exogenous signals. Recent discoveries are shedding light on the mechanisms controlling root development that enable subterranean space exploration [1]. It is largely documented that auxin plays a major role in the control of root growth, even if other hormones such as cytokinin, abscisic acid, brassinosteroids and ethylene have also been implicated in regulating root architecture [2]. Besides, we have recently shown that the phytohormone jasmonic acid also participates in the regulation of sunflower root growth [3]. Besides hormones, novel players involved in root architecture are being discovered, such as oscillating transcriptional regulators that establish branching sites [4].

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; CAld5H, coniferylaldehyde 5-hydroxylase; CCoAOMT, Caffeoyl-CoA O-methyltransferase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DEGs, differentially expressed genes; G, guaiacyl; H, p-hydroxyphenyl; LR, lateral roots; NO, nitric oxide; PR, primary root; SNP, sodium nitroprusside; SNAP, S-nitroso-N-acetyl-DL-penicillamine; S, syringyl; 4CL, 4-coumarate-coenzyme A ligase.

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Nitric oxide (NO) is an inorganic free radical that acts as a signal molecule with different physiological functions. In plants it has diverse roles in disease resistance, senescence, stomatal closure, respiration, xylem differentiation, seed germination, hormone responses, cold response, flowering control and also root development (as reviewed in [5–7]). Studies performed in model species have shown that NO regulates lateral root formation [8], primary root growth [9–11], adventitious roots formation [12] and root hair development [13]. In tomato, the addition of the NO donor sodium nitroprusside (SNP) has been shown to reduce PR length while promoting LR development [8]. Recent evidence indicates that NO is able to produce the inhibition of PR growth through both alterations in PIN1 transporter accumulation, and the consequent deficient polar auxin transport [10], and the reduction of cell division by arresting cells in phase G1 [11]. Thus, a physiological role for NO in the regulation of root architecture is currently accepted although the mechanisms involved are largely unknown. A recent study performed in maize analysing root adaptation to nitrogen fluctuations has demonstrated that NO homeostasis regulates nitrate perception. Moreover, the nitrate-induced root length increase is dependent on a NO signaling pathway [14].

NO can regulate several physiological processes directly by affecting gene transcription [15] or by inducing protein posttranslational modifications [16,17]. In the past years several studies have identified NO-modulated genes in diverse tissues which are involved in different processes such as signal transduction, defence, cell death, transport, primary metabolism and redox regulation [18–22]. Even if the effects of NO on root architecture are recognized, limited information is available on its impact on root gene expression. Badri et al. (2008) [20] performed a large-scale study to evaluate the gene regulation in *Arabidopsis* roots exposed to different signaling compounds, including a NO donor (250 μM SNP). Using a microarray approach it was shown that some of the NO-responsive root genes were different from those differentially expressed in leaves. The NO influence on gene expression was analyzed regarding a defense signaling role of NO. Hence, a transcriptome analysis correlating NO and root architecture is still lacking.

Most of the data related to NO action in plants have been obtained by treatment with exogenous NO donors (SNP, sodium nitroprusside; SNAP, S-nitroso-N-acetylpenicillamine; GSNO, S-nitrosoglutathione). Accumulated evidence indicates that the effects of NO can deeply differ according to the concentration of donor used. For example, applications of high levels of NO have been shown to inhibit plant growth but the application of low levels can promote it [19]. In addition, it is accepted that high NO donor concentrations may induce detoxification responses while lower concentrations might more accurately reflect its role as a signal molecule [23,24]. Alternatively, the question that arises is how the endogenous NO concentration can be mimicked by exogenous NO application if NO concentration in a given tissue is uncertain since its quantitation *in planta* remains difficult. In this complex context, we focused this work on the role of endogenous NO by making use of the NO scavenger cPTIO, a stable free radical that reacts stoichiometrically with NO. cPTIO is widely used to avoid the accumulation of NO in plant tissues and to study [8,10]. Some limits for the use of cPTIO are being described, notably interference for the quantitative determination of NO by electron paramagnetic resonance [25], but it remains a suitable approach to analyze the function of endogenous NO [10,26,27].

Cultivated sunflower is one of the most important sources of vegetable oil worldwide. This crop frequently suffers yield losses originated by root lodging [28] so that studies analysing the basis of root architecture may give important clues to handle this issue. The aim of this work was to analyze the role NO in sunflower root architecture and gene expression using a high throughput approach. Genes whose expression is regulated by endogenous NO were identified taking advantage of the ability of cPTIO as NO quencher. This study reveals lignin metabolism as a target process.

2. Materials and methods

2.1. Plant material and growth condition

Sunflower seeds (*Helianthus annuus* L., Advanta line 10347) were surface-sterilized in 27.5 g/l of sodium hypochlorite for 30 min, rinsed extensively and imbibed in water over night. Imbibed seeds were peeled and allowed to germinate in moistened filter paper for 4 days in a chamber at 25 ± 1 °C and a 14:10 h (light:dark) photoperiod as previously described [3]. The seedlings were then transferred to nutrient solution with or without the supplementation with a nitric oxide donor (25 μM SNP, sodium nitroprusside) (Sigma) or a NO scavenger (700 μM cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) (Invitrogen, Molecular Probes®), and hydroponically grown for 4 days under controlled conditions as indicated above. The nutrient solution content was 3 mM KNO_3, 0.5 mM $MgSO_4$, 1.5 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 25 μM H_3BO_3, 1 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.05 μM Na_2MoO_4, 50 μM FeNaEDTA. The solution was maintained at a constant volume by daily additions.

2.2. Roots length and lateral root number

PR length and LR length were measured from images registered at the end of the treatments using ImageJ 1.38 software (NIH, USA). The number of LR per seedlings was assessed under a magnifying glass. Only LR > 1 mM were counted along the principal root axes from 7 days old seedlings.

All root data are the average of three independent experiments with 6–8 seedlings each one, and the results were analyzed using *t*-Test or Mann–Whitney test.

2.3. Detection of NO

Four old days seedlings were incubated for 18 h with cPTIO or SNP and the NO was monitored by incubating the roots with 12 μM of Diaminofluorescein-FM diacetate (DAF-FM DA) (Invitrogen, Molecular Probes®) in 5 mM MES-KOH, pH 5.7, 0.25 mM KCl, 1 mM $CaCl_2$ for 45 min. Thereafter, the roots were washed three times for 15 min with fresh buffer and examined by epi-fluorescence (excitation 490 nm; emission 525 nm) in an Eclipse E 200 microscope (Nikon, Tokyo).

2.4. RNA isolation and quality controls

For microarray experiments, seedlings were treated for 18 h in nutrient solution or nutrient solution supplied with 700 μM cPTIO. The roots of three independent experiments were harvested and immediately frozen in liquid nitrogen and stored at −80 °C until processing. Total RNA was isolated from 100 mg of frozen tissue using Trizol following the manufacturer's instructions (Invitrogen, Argentina) and quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). The integrity was checked by electrophoresis in 1% agarose gel and the quality was confirmed by RNA 6000 Nano Bioanalyzer (Agilent Technologies, Palo Alto, California, USA) assay.

2.5. Microarrays hybridization

Microarray experiment was conducted as described in [29], using a Sunflower Custom Oligo Microarray (Agilent Technologies)

containing 41013 predicted unigenes derived from Sunflower Unigen Resource v1.0 (http://atgc-sur.inta.gob.ar). The protocol was done in Prince Felipe Research Center, Valencia, Spain. Briefly, 200 ng of total RNA of three independent experiments were used to produce Cyanine 3-CTP labeled cRNA using the Low Input Quick Amp Labelling Kit, One-Colour (Agilent Technologies). Following the 'One-Colour Microarray-Based Gene Expression Analysis' protocol version 6.0 (Agilent Technologies), 2 μg of labeled cRNA was hybridized with a Sunflower Custom Oligo Microarray (Agilent Technologies). Agilent's recommended protocol for microarray workflow quality control was implemented using the Agilent Spike-In Kit.

2.6. Microarrays scanning and data analysis

Slides were scanned in an Agilent Microarray Scanner (G2565BA) according to the manufacturer's protocol. Signal data were collected with Agilent Feature Extraction Software (v9.5.1) following the Agilent protocol GE1_107Sep 09 and the Metric Set GE1_QCMT_Sep09. Data pre-processing was performed using the limma [30] R-package (R Core Team 2012, www.r-project.org). Background was processed using *backgroundCorrec*t function, using the "rma" algorithm. Between arrays normalization was performed using *normalizeBetweenArray* function using "quantile" method. Finally, gene expressions were transformed to log2 scale.

Differential gene expression analysis was performed using a set of *ad hoc* routines to fit a linear mixed effect model gene by gene. Because Sunflower Custom Oligo Microarray includes 4 arrays per chip, the chip effect was included as a random effect. The routines mentioned above were based on the *lme* function of *nlme* library of R (R package version 3.1–105) implemented in InfoStat statistical software (http://www.infostat.com.ar). *p*-Values for contrasts of interest were adjusted by Benjamini–Hochberg procedure [31] as implemented in *mt.rawp2adjp* function of multtest library (R package version 2.10.0). A significant level of 0.001 was applied to select the set of differentially expressed genes. Because of the log2 transformation of gene expressions, contrasts are expressed in log fold-change scale. To express the contrasts differences as fold-change, these were calculated as $2^{(\text{contrast})}$ when the contrast was $\geqslant 0$ and $(-1) \times 2^{-(\text{contrast})}$ when the contrast was <0.

Gene cluster analysis was carried out according to the Gene Ontology terms using Blast2GO v.2.6.0 software [32] or AgriGO v.1.2. [33].

2.7. Quantitative RT-PCR (qRT-PCR)

Specific primer sets for qRT-PCR were designed based on selected sequences using Primer Express 3.0 software (PE Applied Biosystems, USA) and are listed in Supplementary Table S3. DEGs belonging to different functional categories were selected at random for primer design. RNA was extracted with the RNAeasy Plant Mini Kit (Qiagen) and first-strand cDNA was reverse transcribed from DNase treated RNA according to manufacturer instructions (Invitrogen, Argentina). The real time RT-PCR reactions were performed in a 20 μl volume containing 10 μl Power SYBR Green PCR Master Mix (Applied Biosystems, UK), 500 nm of each primer and 4 μl of the corresponding cDNA dilution. The PCR reactions were run in Applied Biosystems Step OneTM Real Time PCR Systems, using the following program: 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 61 °C for 1 min. Specificity of amplicons was verified by identification of a single peak in the melting curve analysis. Control PCRs with no template and minus RT-PCR were performed for each primer pair. The qRT-PCR assay was carried out using two biological replicates for each treatment and three technical replicates for each biological replicate, derived from independent cDNA synthesis. The sunflower reference genes previously characterized, actin (GenBank: FJ487620.1) [34] and EF-1α (GenBank: AAM19764.1) [35] were used as endogenous control for expression level. Quantifying the relative changes in gene expression was performed using the $2^{-\Delta\Delta CT}$ method.

2.8. Lignin analyses

Cell walls were prepared from dried root tissues. Briefly, dried roots were ground into fine powder and homogenized with water. Then, they were centrifuged (3000*g*, 10 min) and the pellet was washed by successive stirring and centrifugation as follows: twice with Triton X-100, once with water, three times with ethanol 100% and three times with diethyl ether. The pellet was dried in a vacuum system [36].

Lignin quantification of the cell walls was performed using acetyl bromide, as described in [37]. Alkaline nitrobenzene oxidation of lignifying cell walls and HPLC analyses were performed essentially as described [38]. The quantification of *p*-hydroxybenzaldehyde, vanillin and syringaldehyde was performed at 290 nm using the corresponding standards. Thioacidolysis of lignifying cell walls, which solubilizes the β-*O*-4 lignin core, and GC–MS analyses were performed [38] using a Thermo Finnigan Trace GC gas chromatograph, a Thermo Finnigan Polaris Q mass spectrometer and a DB-XLB, J&W (60 × 0.25 mM I.D.) column. Mass spectra were recorded at 70 eV.

3. Results

3.1. Endogenous NO regulates sunflower root architecture

To analyze the effect of NO on sunflower root architecture, 3 days-old seedlings were further grown in the presence of NO donors or the NO scavenger cPTIO. Treatments where performed during 4 days, the time required to detect statistically significant phenotypical differences. Fig. 1 illustrates that 25 μM SNP clearly decreased primary root (PR) length without changing the general root architecture. This effect of SNP was dose dependent in concentrations ranging from 5 to 25 μM and higher concentrations could not be tested since they produce a severe browning of the root system (not shown). Treatment of the seedlings with 60 μM SNAP, another NO donor, also produced PR growth inhibition (Fig. 1). On the other hand, 700 μM cPTIO deeply affected root architecture, mostly modifying the lateral root (LR) pattern (Fig. 1). It can be seen that the treatment prevented the occurrence of new LR. This finding confirms the requirement of NO to promote LR development observed in tomato and attributed to its participation in the auxin signaling pathway involved in the formation of LR primordia [8]. Additionally, in the basal zone of the roots, limiting with the hypocotyl, LR appeared longer compared to the control (Fig. 1). This observation suggests that they might correspond to those primordia formed before cPTIO addition. Interestingly, the length of these LR was increased in the presence of cPTIO, highligting a growth estimulation by NO depletion. Finally, it can be seen that seedlings growing in the presence of cPTIO showed a thinner PR in the zone near the tip (Fig. 1).

The effectiveness of cPTIO as NO scavenger was assessed using the cell-permeable fluorescent probe Diaminofluorescein-FM diacetate (DAF-FM DA) for the detection of NO. Sunflower roots growing under different treatments were incubated with DAF-FM DA, and evaluated by fluorescence microscopy. NO was detected in the tip of control roots growing in nutrient solution as well as in SNP treated seedlings used as positive control (Fig. 2A). On the other hand, roots grown with 700 μM cPTIO showed decreased endogenous levels of NO as evidenced by undetectable probe fluorescence in Fig. 2A. Relative quantification of DAF fluorescence

Fig. 1. NO is involved in determining sunflower root architecture. Three days old sunflower seedlings were transferred to nutrient solution (control) or nutrient solution supplemented with the nitric oxide donors sodium nitroprusside (SNP, 25 μM) and S-nitroso-N-acetyl-DL-penicillamine (SNAP, 60 μM), or the NO scavenger carboxi-PTIO (cPTIO, 700 μM) and grown for 4 days. A representative photograph of 7-day-old seedlings is shown. Bar = 1 cm.

of several repetitions confirmed the reduction of NO levels in roots growing in the presence of cPTIO, while root tips supplemented with SNP exhibit almost a threefold increase compared to control ones (Fig. 2B).

To focus on the role of endogenous NO, a deeper analysis of the effect of NO depletion on the phenotype of sunflower roots was performed. Fig. 3A confirms that cPTIO treated roots did not show a significant change in PR length when compared to untreated control seedlings, but LR length and number were clearly affected (Fig. 3B, C). LR length increased by 95% while a reduction of 65% in the number of LR per seedling was observed after NO depletion. Globally, the total length of LR is reduced by 27% upon cPTIO treatment. In conclusion, our results indicate that endogenous NO may be required for LR development, as previously observed in tomato [8], but may also participate in the definition of LR length in sunflower seedlings.

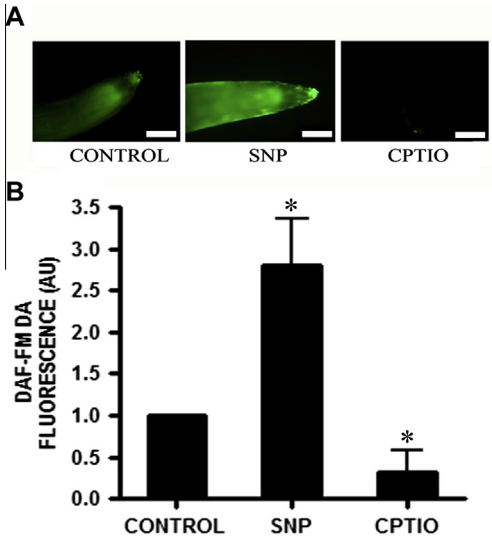

Fig. 2. cPTIO reduces NO levels in root tips. Four days old sunflower seedlings were treated with 25 μM SNP or 700 μM cPTIO for 18 h. After treatment, the roots were loaded with 12 μM DAF-FM DA for 30 min and observed by epifluorescence microscopy (magnification: 100×). Bars = 0.2 mm. (A) Root tips of control, SNP and cPTIO treatment, respectively. (B) Quantification of fluorescence in the root tips as described in materials and methods. Data are expressed as arbitrary units relative to control. The mean ± SD of 3 independent experiments performed in triplicate is shown. Asterisks indicate a significant difference compared to control seedlings ($p \leqslant 0.05$).

3.2. Transcriptional regulation by endogenous NO in sunflower roots

A 60-mer oligo microarray representing 41013 predicted unigenes from sunflower was used to characterize root-specific genes affected by cPTIO treatment. In order to detect early responses to the treatment seedlings were treated for 18 h with 700 μM cPTIO followed by RNA extraction from the roots. Labeled cRNAs were hybridized on a validated Agilent platform [29]. Expression ratios between control and cPTIO treated samples showed that several genes were differentially expressed with a stringent p value ($p \leqslant 0.001$) (Supplementary Table S1). Hence, 250 genes were over-expressed in the roots in response to cPTIO and they are candidates to be down regulated by NO *in planta*. On the other hand, 80 genes were down regulated and may be up regulated by endogenous NO. Together, 330 out of 41013 unigenes were differentially expressed genes (DEG) in sunflower roots (~0.80%).

In order to validate the results obtained in the microarray assays several DEGs were analyzed by quantitative real time PCR (qRT-PCR) using specific primers detailed in Supplementary Table S3. DEGs representing different functional categories were selected at random. Table 1 shows that the effect of cPTIO treatment on the expression levels detected by qRT-PCR was consistent with the results of chip hybridization. Similar results were obtained by using actin or elongation factor EF-1α as reference genes in the qPCR (not shown).

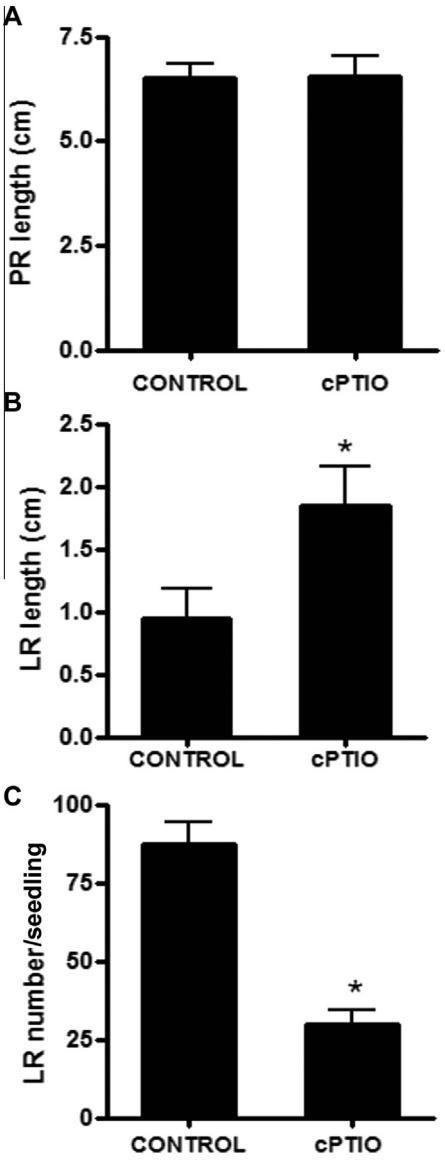

Fig. 3. NO depletion increases lateral root (LR) length and decreases LR number in sunflower seedlings. (A) Primary root length, (B) lateral root length and (C) lateral root number, were measured in sunflower seedlings grown for four days in nutrient solution (control) or nutrient solution supplied with 700 μM cPTIO. Data shown represent means ± s.d. ($n = 6$) for three different experiments. Asterisks indicate a significant difference compared to control seedlings ($p \leqslant 0.05$).

The DEGs upon cPTIO treatment were further classified by Gene Ontology (GO) terms using the Blast2GO software [32] even if only 75% of them present an associated GO number for biological process, cellular component or molecular function. Fig. 3 is a multilevel pie plot for biological process that shows that the DEGs belong to several functional categories. The complete list of genes included in each category of biological process is presented in Supplementary Table S2. As seen in Fig. 4A, among the up-regulated genes upon cPTIO treatment, the functional categories over-represented are electron transport (including genes for peroxidases, cytochrome P450, glutathione reductases) and drug transmembrane transport (e.g. ABC transporters, multidrug resistance, mate efflux proteins). Other categories are also well represented with at least 11 genes induced. They are oxidation/reduction (e.g. aldo keto reductases, cinnamyl alcohol dehydrogenases), monocarboxylic acid metabolic process and carboxylic acid biosynthetic process (e.g. 4-coumarate-coA ligase, cinnamyl alcohol dehydrogenase, 12-oxophytodienoate reductase, isocitrate lyase). It is noteworthy that monocarboxylic acid and carboxylic acid metabolic process include several genes related with the secondary metabolism. Concerning the down-regulated genes by cPTIO treatment (Fig. 4B), the different GO appeared represented in approximately equal proportions including, among others: response to chemical stimulus and electron transport. Similar results were obtained by classifying the DEGs with AgriGO tools, designed for gene ontology analysis focused on agricultural species [33] (not shown).

Table 1
Comparison of gene expression levels obtained by cDNA microarrays and qRT-PCR analysis for 13 differentially expressed genes in roots upon cPTIO treatment (selected from Supplementary Table S1). Actin, GenBank: FJ48720.1, was used as a reference gene in qRT-PCR reactions.

ID	Gene description	Fold change	
		Microarray	qRT-PCR
HeAn_C_719	Glutathione S-transferase	10.31	75.09
HeAn_S_35143	Glutathione reductase	2.18	6.89
HeAn_S_14756	Cinnamyl alcohol dehydrogenase	62.37	963.9
HeAn_S_14757	Cinnamyl alcohol dehydrogenase	32.94	472.7
HeAn_S_38530	Aldo keto reductase	20.96	20.35
HeAn_C_10091	Mate efflux family protein	6.06	7.44
HeAn_C_8675	Cytrocrome p 450	4.35	6.63
HeAn_C_3995	Iaa-aminoacid hydrolase	2.1	2.6
HA_S_21226	Nac domain protein. NAC TF	3.03	2.37
HeAn_C_6167	Nitrate transporter	10.66	5.14
HeAn_C_2762	Class III peroxidase	7.53	6.33
HeAn_S_14421	ER33	−1.99	−2.33
HeAn_C_4816	Nitrate transporter	−1.51	−2.1

3.3. Lignin biosynthesis appears as a central target of gene regulation by NO

To further understand the putative function of the DEGs regulated upon cPTIO treatment, they were mapped in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using Blast2GO v.2.6.0 software. This analysis revealed several pathways affected but stressed the importance of genes related with the secondary metabolism highlighting the phenylpropanoid biosynthesis, and particularly lignin biosynthesis. Table 2 presents the list of genes involved in lignin biosynthesis whose expression was modified in roots depleted of NO. It includes genes related to monolignol biosynthesis and polymerization such us 4-coumarate-coenzyme A ligase (4CL), cinnamyl alcohol dehydrogenases (CAD), coniferylaldehyde 5-hydroxylase (CAld5H), Caffeoyl-CoA O-methyltransferase (CCoAOMT), S-adenosylmethionine-dependent methyltransferase (like COMT) and several peroxidases. All of these peroxidases were identified as Class III (extracellular) according to their Enzyme Commission number (EC:1.11.1.7) and the PeroxiBase [39], and could hence be involved in lignin polymerization. Most of these lignin-related genes were up-regulated by cPTIO treatment and only three were down-regulated (two peroxidases and a CAD). Particularly striking is the high induction observed for two cinnamyl alcohol dehydrogenase genes (HeAn_S_14756, HeAn_S_14757) (Table 2). In conclusion, the expression analysis performed points to endogenous NO as a transcriptional regulator of the lignin biosynthetic process in sunflower roots.

3.4. Lignin composition is influenced by NO in sunflower roots

Microarray experiments revealed that cPTIO treatment influences the expression of lignin biosynthetic genes which suggests that NO depletion could alter lignin content and/or composition in sunflower roots. Lignin, an integral part of the secondary cell

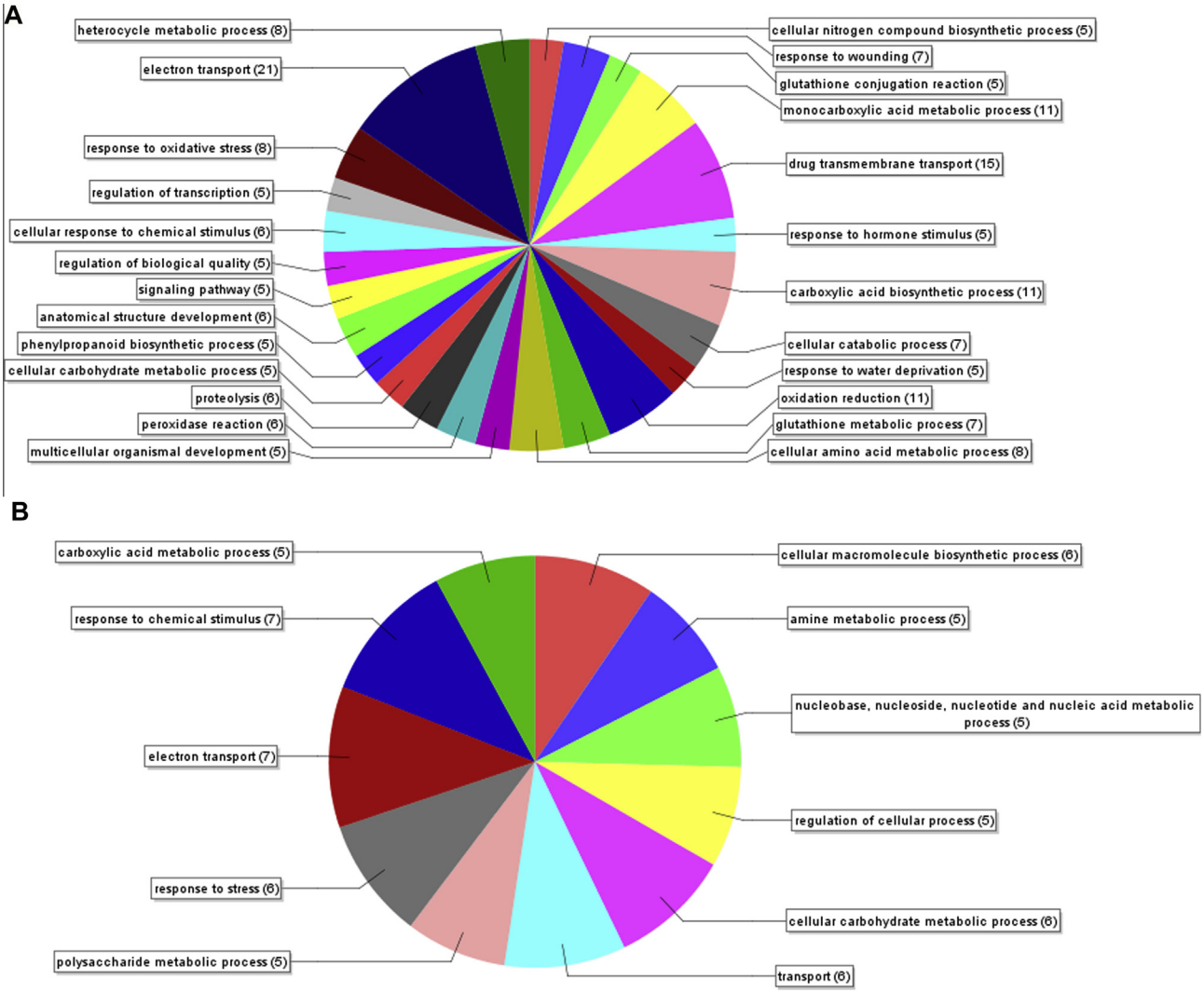

Fig. 4. NO depletion affects gene expression in sunflower roots. Blast2GO multilevel pie graphs for biological process category were used to analyze the genes differentially expressed ($p \leqslant 0.001$) in sunflower seedling roots in response to cPTIO (700 μM) treatment. Multilevel pies graphs for biological process (filtered by n° of sequences: cut off = 5) are shown for (A) up-regulated genes ($n = 189$) and (B) down-regulated genes ($n = 60$). Some DEGs are not represented in these graphs because only 75% of them have an associated GO number for biological process.

Table 2
Genes related to lignin biosynthesis differentially expressed in sunflower roots in response to cPTIO treatment (selected from Supplementary Table S1).

ID	Gene description	Fold change
HeAn_C_6385	4-coumarate-coenzyme A ligase	1.40
HeAn_S_16106	4-coumarate-coenzyme A ligase	1.58
HeAn_S_14756	Cinnamyl alcohol dehydrogenase	62.37
HeAn_S_14757	Cinnamyl alcohol dehydrogenase	32.94
HeAn_S_34967	Cinnamyl alcohol deshidrogenase	−1.70
HeAn_S_14857	Cinnamyl alcohol dehydrogenase-like protein	7.17
HeAn_C_2021	Cinnamyl alcohol dehydrogenase-like protein	7.21
HeAn_C_2343	s-Adenosylmethionine-dependent methyltransferase	3.21
HeAn_C_9413	Caffeoyl-CoA o-methyltransferase-like protein	12.13
HeAn_C_7556	Coniferylaldehyde 5-hydroxylase	3.58
HeAn_C_1942	Class III peroxidase	4.25
HeAn_S_38835	Class III peroxidase	1.28
HeAn_S_20213	Class III peroxidase	3.04
HeAn_C_2762	Class III peroxidase	7.53
HeAn_S_28827	Class III peroxidase	2.06
HeAn_C_1509	Class III peroxidase	−2.71
HeAn_C_1510	Class III peroxidase	−2.38

walls, is a polymeric material mainly composed of phenylpropanoid units derived from three cinnamyl alcohols (monolignols): *p*-coumaryl, coniferyl, and synapyl alcohols giving rise to the hydroxyphenyl (H), guaiacyl (G) and synapyl (S) lignin polymers, respectively (for review, see [40]). Seedlings treated with cPTIO during 4 days were first evaluated microscopically for lignin content and distribution. Cross sections of PR stained with phloroglucinol showed a similar distribution of vessels in vascular bundles in cPTIO-treated plants and control ones and no differences in total lignin content were detected (data not shown). In a more exhaustive analysis, the total lignin content of the root cell walls was measured using acetylbromide, a commonly employed method for lignin determination [36,41]. Table 3 shows that lignin content remained unchanged in roots treated with the NO scavenger cPTIO. Lignin levels of around 13 μg per mg of cell walls were determined, similar to those obtained in tomato roots [42]. The lignin monomeric composition of root cell walls was further determined by nitrobenzene oxidation (NBO) and thioacidolysis (TAC). Thioacidolysis acts through the cleavage of β-*O*-4 linkages, i.e. the linear polymer, so it may be a limiting technique when the sample is enriched in C—C bonds. β-*O*-4 linkages are mainly formed in the so called end-wise polymerization, however, in the bulk polymerization a

Table 3
Changes in lignin content and monomeric composition in sunflower roots in response to cPTIO treatment.

	Lignin content (μg/mg CW)	Relative monomeric composition (%)							
		H-units		G-units		S-units		G/S Ratio	
		NBO	TAC	NBO	TAC	NBO	TAC	NBO	TAC
CONTROL	13.86 ± 0.73	6	–	55.5	67	38.5	33	1.44	2.03
cPTIO	13.76 ± 0.70	8.5	–	60	79	31.5	21	1.90	3.76

Lignin content was measured by acetyl bromide. Lignin monomeric composition was revealed by analysis of nitrobenzene oxidation (NBO) and thioacidolysis (TAC) of root cell walls products from control and 700 μM cPTIO treated plants (4 days of treatment). H-units: hydroxyphenyl units; G-units: guaiacyl units; S-units: syringyl units; CW: cell walls.

highly branched and condensed lignin polymer, with carbon–carbon bonds, is formed. Hence, the differences found between NBO and thioacidolysis results may be attributed to the diverse targets of the depolymerization techniques, meaning the analyzed cell walls have a very condensed structure. Table 3 shows that despite differences revealed by each method, both of them revealed changes in lignin composition. cPTIO treatment increased the relative amount of guaiacyl (G) units yielding a higher G/S ratio (Table 3).

4. Discussion

Root architecture results from a complex control of root growth and development that enables adaptation to diverse environmental conditions. In this report, we present evidence on the participation of endogenous NO in sunflower root organogenesis simultaneously to the regulation of gene expression, deeply affecting lignin metabolism. This conclusion is supported by a phenotypic evaluation together with a large-scale transcriptional analysis of roots depleted of endogenous NO by the application of cPTIO. Roots treated with this scavenger revealed an altered pattern of LR and a thinner PR confirming that NO modulates sunflower root branching and probably deep soil exploration. It must be emphasized that the density of LR was reduced in the presence of cPTIO meaning that NO plays a central role in determining root ramification. On the other hand, endogenous NO might inhibit LR length since this work shows an extensive elongation of preformed LR when cPTIO treatment was applied. In the same line of evidence, a decrease in LR length has been observed in tomato seedling exposed to increasing concentrations of a NO donor [8], suggesting that the inhibition of LR elongation by NO might be a general mechanism operating in plants. Concerning PR length, we failed to detect significant changes in treated sunflower seedlings, an effect previously reported for tomato plants [8]. However, and in accordance to our data, not significant elongation of the PR was observed in *Arabidopsis* plants submitted to cPTIO treatment [43], probably reflecting a species specific fine tuning response to endogenous NO concentration.

A link between lignin and plant growth is currently accepted. Accumulated evidence indicates that certain lignin-deficient mutants often show reduced growth [44,45], even if the mechanism that connects altered monolignol biosynthesis and plant growth inhibition still remains unknown [46]. It is accepted that lignin strengthens the cell walls but the functional significance of lignin composition remains under debate. It has been suggested that a lignin polymer containing sinapyl alcohol units may have superior mechanical support properties [47]. The variation in lignin composition suggests a precise functional adaptation; thus, the more linear S lignin might provide herbaceous angiosperms a strong yet flexible polymer [40].

In agreement, the maize S lignin deficient mutants bm3, disrupted in the caffeic acid O-methyltransferase (COMT) gene, present cell walls of impaired vascular integrity, a loss of flexibility and increased susceptibility to lodging [48]. Our results suggest that a fine adjustment of NO levels in the roots would be critical to regulate lignin composition. Therefore, it can be suggested that the high lignin G/S rate obtained at low NO concentrations would determine a more crosslinked lignin structure, more susceptible to rupture, while higher NO levels may lead to a decrease in the G/S ratio and more resistant and flexible tissues. Although this concept correlating lignin composition and function has been originally developed for aerial tissues (stem), a relationship between lignin and root architecture is emerging. The rice transcription factor OsNAC9 has been implicated in the definition of root architecture regulating the expression of key genes, including a cinnamoyl CoA reductase involved in lignin biosynthesis [49]. Additionally, the *Medicago truncata* mutant *cra1* (Compact Root Architecture1) shows compact root architecture with short and thick roots. CRA1 gene regulates lignification and cra1 roots have a reduction in lignin levels [50]. Associations between lignin deposition and changes in the root architecture of *Arabidopsis* have also been detected in plants exposed to copper excess [51]. Together, accumulated evidence suggests a tight relationship between root growth and lignin content.

In this report a microarray approach was used to evaluate the ability of endogenous NO to regulate gene transcription in root seedlings. The analysis allowed to identify 330 DEG following cPTIO treatment, even if a stringent p value was imposed ($p \leqslant 0.001$). Consistent results were found compared to the data reported by Badri et al. [20], who performed a transcriptomic analysis of *Arabidopsis* roots treated with the NO donor SNP. NO application resulted in a high number of repressed genes and a few induced genes. An equivalent situation was found in this work studying the effect of NO depletion, since the number of induced genes triplicates that of repressed genes. Even if the meaning of this finding is uncertain, both approaches point out to NO mainly acting as a repressor of gene expression in roots. Several DEGs detected correspond to functional groups related to NO signaling in other species and different organs [19–21]. As expected, genes involved in oxidation/reduction were strongly affected by cPTIO treatment, in accordance with previous reports showing that NO affects the redox homeostasis of root cells [52,53]. Interestingly, the gene functional categories affected in roots by NO supply [20] or NO depletion (this work) are roughly the same, with an inverse correlation between induced and repressed clusters depending on NO availability. For example, while a repression of genes involved in the biosynthesis of phenylpropanoids was observed after SNP treatment in *Arabidopsis* roots [20], an induction is reported here in sunflower after NO depletion.

Consistent with the phenotype observed, several genes putatively related to root growth and development were differentially expressed in NO depleted sunflower roots (Supplementary Table S1). Among them there are genes involved in DNA replication (regulator of chromosome condensation, DNA gyrase, cytoskeleton organization proteins) and cell wall related (cellulose synthases, pectinestearase, peroxidases, and several lignin biosynthetic

genes). Nevertheless, the most prominent data reported here is that lignin metabolism is a central target of gene regulation by NO depletion. Roots treated with the NO scavenger showed increased RNA levels of several enzymes involved in lignin biosynthesis and we demonstrate that this induction correlates with a differential composition of lignin leading to an increased G/S relationship.

NO participates in lignification processes in different ways. Direct effects of NO on hemeproteins involved in lignin biosynthesis have been demonstrated [54,55]. In fact, NO donors are capable of inhibiting the activity of peroxidases [54]. In addition, indirect effects of NO related to lignification have been proposed, including transcriptional activation of certain genes, control of H_2O_2 levels (used by peroxidases for the polymerization of hydroxycinnamyl alcohols) and the modulation of the enzymes involved in its removal (i.e. ascorbato peroxidase) [56,57]. SNP treatments have also shown to modify lignin content and the activity of lignin related enzymes in soybean seedlings [58].

Lignins are heteropolymers which result from the oxidative coupling of three different monolignols, in a reaction mediated by peroxidases. In angiosperms these polymers are largely composed of G and S units [59]. Here, we show that cPTIO treatment increases the G/S ratio without changing the total content of lignin. This means that physiological concentrations of NO in the roots would regulate the expression of certain genes involved in lignin biosynthesis leading to a polymer enriched in S-monolignols.

Despite the increment in CAld5H expression observed upon NO depletion, no accumulation of the main final products of this enzyme was detected (sinapyl alcohol, sinapylaldehyde or sinapic acid). These data could be justified by the existence of a non studied metabolic sink (i.e. 5-hydroxyconiferyl alcohol, incorporated to lignins as benzodioxane structures). In this scenario, another possibility emerges that could justify an increase in the lignin G/S ratio when NO is scavenged. According to previous reports, a main role for NO in the regulation of peroxidases could determine a selective polymerization. Only basic peroxidase isoenzymes are able to oxidize syringyl compounds to ultimately form S units [60]. Specifically, ZePrx, a basic peroxidase that has been unequivocally linked to lignin biosynthesis [61], is up-regulated upon NO addition [62]. Hence, basic peroxidases could be down-regulated by NO depletion, reducing S lignin levels. Interestingly, previous evidence showed that NO inhibits the activity of a coniferyl alcohol peroxidase from *Zinnia elegans* which polymerizes G units [61]. This observation is in agreement with the relative increase in the content of G-lignin found in the cPTIO-treated cell walls. Even if peroxidase isoenzymes involved in lignin biosynthesis are not yet identified in sunflower, this analysis supports the involvement of peroxidases in the NO-regulated lignin polymerization.

The structural differences between G and S lignin are caused by the presence of the methoxyl group at the 5-position, which results in S lignin being more linear and less crosslinked [40].

This report provides the first evidence on a modulation of lignin composition by NO during root growth. Even if a direct relationship between levels or composition of lignin and root architecture has not been established, the relevance of these findings deserves future investigation to elucidate their impact on root lodging in sunflower crops.

5. Conclusions

Depletion of endogenous NO by using the scavenger cPTIO revealed that NO is involved in the definition of sunflower root architecture. While the primary root length was not affected by cPTIO treatment, the number of lateral roots was reduced and their length increased, deeply affecting root branching. Transcriptomic changes induced by cPTIO include genes from several functional categories and pathways, notably the phenylpropanoid biosynthesis. Several genes involved in lignin biosynthesis were up-regulated and we demonstrate that cPTIO treatment results in an altered composition of lignin, a component of the secondary wall of plant cells. Hence, endogenous NO affects lignin structure in the roots.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.niox.2014.04.004.

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