PCP
PLANT & CELL PHYSIOLOGY

Regular Paper

Differential Transcriptomic Analysis by RNA-Seq of GSNO-Responsive Genes Between Arabidopsis Roots and Leaves

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(Received May 6, 2013; Accepted February 27, 2014)

S-Nitrosoglutathione (GSNO) is a nitric oxide-derived molecule that can regulate protein function by a post-translational modification designated *S*-nitrosylation. GSNO has also been detected in different plant organs under physiological and stress conditions, and it can also modulate gene expression. Thirty-day-old Arabidopsis plants were grown under hydroponic conditions, and exogenous 1 mM GSNO was applied to the root systems for 3 h. Differential gene expression analyses were carried out both in roots and in leaves by RNA sequencing (RNA-seq). A total of 3,263 genes were identified as being modulated by GSNO. Most of the genes identified were associated with the mechanism of protection against stress situations, many of these having previously been identified as target genes of GSNO by array-based methods. However, new genes were identified, such as that for methionine sulfoxide reductase (MSR) in leaves or different miscellaneous RNA (*miscRNA*) genes in Arabidopsis roots. As a result, 1,945 GSNO-responsive genes expressed differently in leaves and roots were identified, and 114 of these corresponded exclusively to one of these organs. In summary, it is demonstrated that RNA-seq extends our knowledge of GSNO as a signaling molecule which differentially modulates gene expression in roots and leaves under non-stress conditions.

Keywords: Arabidopsis • Massively parallel sequencing • Methionine sulfoxide reductase • Nitric oxide • Plant signaling mechanisms • RNA-seq • *S*-nitrosoglutathione.

Abbreviations: FNR, ferredoxin-NADP reductase; FPKM, fragments per kilobase of exon per million fragments mapped; Fc, fold change; GO, gene ontology; G6PDH, glucose-6-phosphate-1-dehydrogenase; GSH, glutathione; GSNO, *S*-nitrosoglutathione; HR, hypersensitive response; LRR, leucine-rich repeat; *miscRNA*, miscellaneous RNA; MetSO, methionine sulfoxide; MSR, methionine sulfoxide reductase; NO, nitric oxide; NO_2-Tyr, 3-nitrotyrosine; PTM, post-translational modification; qRT–PCR, quantitative real-time reverse transcription–PCR; ROS, reactive oxygen species; RLP, receptor-like protein; RNS, reactive nitrogen species; SNP, sodium nitroprusside; TSS, transcription start sites.

Introduction

Nitric oxide (NO) is reportedly involved in several physiological or stress-response processes in plants. Furthermore, there is growing evidence that NO and reactive nitrogen species (RNS) can mediate post-translational changes through mechanisms such as *S*-nitrosylation or tyrosine nitration of proteins. Accordingly, significant numbers of proteins have been identified as NO targets in plants in recent years (Lindermayr and Durner 2007, Chaki et al. 2009b, Lozano-Juste et al. 2011, Astier et al. 2012, Chaki et al. 2013, Begara-Morales et al. 2013a, Begara-Morales et al. 2013b, Kato et al. 2013, Mengel et al. 2013). *S*-nitrosoglutathione (GSNO) is a major low molecular weight *S*-nitrosothiol that is considered to be an endogenous reservoir of NO in cells (Leitner et al. 2009) and it has been recently identified and quantified in plants under natural and stress conditions (Airaki et al. 2011a, Leterrier et al. 2012). GSNO is phloem mobile so that is considered to be a vehicle of NO for long distances, this being important for the redox signaling mechanisms (Malik et al. 2011). GSNO is decomposed to oxidized glutathione (GSSG) and NH_3 by an NADH-dependent *S*-nitrosoglutathione reductase (GSNOR), and consequently this enzyme can regulate the GSNO content under physiological and stress conditions (Feechan et al. 2005, Barroso et al. 2006, Rusterucci et al. 2007, Lee et al. 2008, Leterrier et al. 2011, Corpas et al. 2013, Xu et al. 2013). In addition, GSNO can carry out transnitrosylation reactions in which an

Plant Cell Physiol. 55(6): 1080–1095 (2014) doi:10.1093/pcp/pcu044, available online at www.pcp.oxfordjournals.org

NO group is transferred from an *S*-nitrosothiol to a cysteine-thiol group of a target protein. For that reason, GSNO has been used to study *S*-nitrosylation of different proteins *in vitro* and it has been related to plant responses against biotic stress (Feechan et al. 2005, Chaki et al. 2009a). The number of *S*-nitrosylated proteins has grown significantly, including some *S*-nitrosylated proteins which have been studied at the molecular level (Wang et al. 2009, Palmieri et al. 2010, Astier et al. 2011, Yun et al. 2011, Begara-Morales et al. 2013b, Kato et al. 2013, Begara-Morales et al. 2014). In addition, NO signaling through protein post-translational modifications (PTMs) and NO-dependent signaling mechanisms based on changes in gene expression are also part of the signaling pathway. There is evidence that NO acts as a signaling molecule during biotic stress in plants (Delledonne et al. 1998, Durner et al. 1998, Krasylenko et al. 2010).

Some years ago, the emergence and development of microarray technology allowed researchers to conduct large-scale studies, analysing the response of thousands of genes in a single experiment. This technology has been used to analyze NO-responsive genes in leaf, cell cultures and roots of *Arabidopsis thaliana* (Polverari et al. 2003, Parani et al. 2004, Badri et al. 2008, Ahlfors et al. 2009), and other plant species (for a review, see Besson-Bard et al. 2009). Taken together, these studies indicate that a high percentage of NO-responsive genes are related to oxidative stress in response to different stress conditions and that they are gene-encoding proteins involved in signal transduction such as kinases and phosphatase proteins (Grün et al. 2006, Besson-Bard et al. 2009). Most of these studies have used sodium nitroprusside (SNP) as an NO donor, while GSNO has been used only to analyze the NO-dependent transcriptomic response in *Medicago truncatula* plants (Ferrarini et al. 2008). Thus, NO-responsive genes have been identified using medium- and large-scale transcriptomic analyses including cDNA amplified fragment length polymorphism (AFLP) and microarray technology (for a review, see Besson-Bard et al. 2009). However, in recent years, new high-throughput sequencing methods, termed massively parallel sequencing or RNA-seq, have emerged as useful tool that could replace and improve existing methods because of their advantages over array-based methods (Wilhelm and Landry 2009, Van Verk et al. 2013): (i) it is not necessary to have previous knowledge of the transcribed regions, so that it is easier to carry out gene expression studies in complex organisms; (ii) RNA-seq technologies permit not only a gene expression quantification, but also the identification of different isoforms, promoters, transcription start sites (TSS) or sites of alternative splicing at the same time; and (iii) RNA-seq output is at the theoretical maximum of base pair resolution. As a result, RNA-seq data have very recently started to be compiled in higher plants (Lee et al. 2010, Donà et al. 2013, de Cremer et al. 2013, Postnikova et al. 2013, Van Moerkercke et al. 2013).

The aim of the present study is to analyze the differential expression of the GSNO-responsive genes between roots and leaves under non-stress conditions using Arabidopsis as the model plant. This large-scale gene expression analysis has been performed using the paired-end RNA-seq technology developed by Illumina, this being the first available report in plants to use this approach in order to understand the GSNO signaling mechanism between different organs in plants. This allowed us to identify genes and biological pathways that respond to GSNO signaling which previously were not identified by array-based methods in plants.

Results

Fig. 1A shows the appearance of 30-day-old Arabidopsis plants and the experimental design used to apply three treatments to the roots in independent plants: 1 mM GSNO (NO donor), 1 mM glutathione (GSH) and distilled water (control). **Fig. 1B** schematically illustrates the process flux of the experimental procedure. Thus, leaves and roots from each treatment were harvested and used for total RNA isolation. Then, paired-end libraries were prepared and sequenced as described in the Material and Methods. The quality of the data and of the generated sequences was checked using the Fast QC software and Phred measure Score, respectively. The high quality of the data generated is shown in **Table 1**. The first column lists the total fragments generated, which is the sum of sequencing in both directions. The percentage of high-quality fragments is >80% in all cases (20 units or more in Phred values which corresponds to a sequencing error rate of 1%).

The alignment vs. the Arabidopsis sequence was performed using TopHat (Trapnell et al. 2009) and Bowtie (Langmead et al. 2009) software. Following this, we used the Cufflinks program (Trapnell et al. 2010), which provides relative abundance values by calculating fragments per kilobase of exon per million fragments mapped (FPKM) (Mortazavi et al. 2008, Mizrachi et al. 2010). This program is also used to find different isoforms, promoters, TSS or sites of alternative splicing (**Table 2**). In this study, a sequenced read was employed in the analysis when the average quality vs. the reference alignment was >20 units, and the minimum number of fragments aligned in a given locus was 200 in both sequencing directions. Moreover, a false discovery rate (Benjamini–Hochberg FDR) of 0.01 was used to reduce false positives. Furthermore, to eliminate background noise, we set an arbitrary threshold of 80 FPKM, which was determined by comparing the expression of different housekeeping genes that were used as an internal expression control. Therefore, we assumed that a gene with an FPKM value >80 is expressed, while an FPKM value <80 indicates that the gene is not expressed.

GSNO-responsive genes in Arabidopsis plants

The treatment of roots of Arabidopsis plants with 1 mM GSNO prompted expression changes in 3,263 genes in the whole plant. Using gene ontology (GO) annotations from the TAIR database, the functional annotation of these genes is shown in **Fig. 2**, where 2,799 sequences were assigned to molecular function

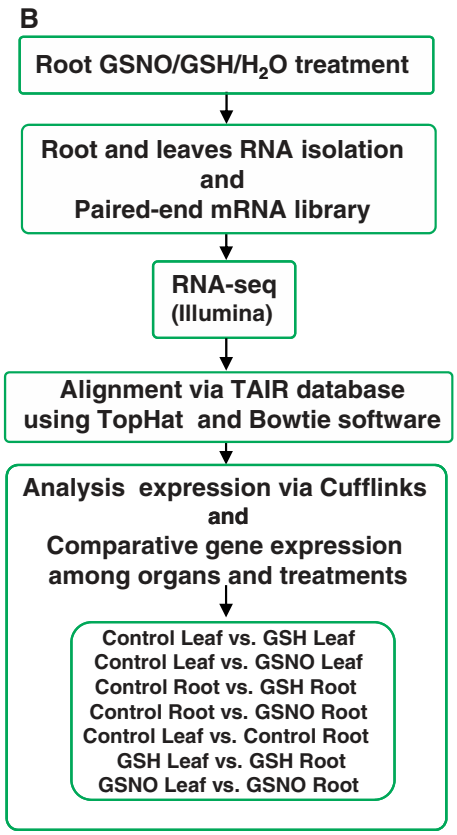


Fig. 1 (A) Appearance of a 30-day-old Arabidopsis plant and the *in vitro* experimental design used to apply three different treatments to the roots: 1 mM GSNO (NO donor), 1 mM GSH or distilled water (control). After 3 h of each treatment, leaves and roots were harvested and used for RNA isolation. (B) Flow chart of the experimental procedure.

(**Fig. 2A**), 2,869 sequences were assigned to biological process (**Fig. 2B**) and 2,927 sequences were assigned to the cell component (**Fig. 2C**).

To determine the potential GSNO signaling mechanisms between roots and leaves, we also compared RNA-seq results found in these organs after different root treatments with

Table 1 Total number and percentage of high quality fragments obtained in both directions of sequencing by RNA-seq of root and leaf samples of 30-day-old Arabidopsis plants in which the roots were incubated for 3 h either with solutions of 1 mM GSNO (a NO donor) and 1 mM GSH or with distilled water (control)

Sample	Total number of reads	% High quality fragments
Control root	64,387,174	86.7/85.1
GSH root	72,544,680	86.6/88.4
GSNO root	55,118,116	91.3/92.8
Control leaf	72,134,580	85.0/87.1
GSH leaf	72,137,204	90.1/91.3
GSNO leaf	71,633,600	87.9/89.0

Percentage high quality fragments refers to the Phred value which is widely accepted to characterize the quality of DNA sequences, and used to evaluate the efficacy of the sequencing method.

Table 2 Summary of the most significant results (number of genes, isoforms, promoters, sites of alternative splicing and transcription start sites) obtained by RNA-seq analysis using the Cufflinks program

Comparison	Genes	Iso forms	Pro moters	Splicing	TSS
Control root vs. GSH root	4,081	4,110	173	411	3,266
Control root vs. GSNO root	1,715	1,743	84	265	1,446
Control leaf vs. GSH leaf	2,900	2,898	97	203	2,397
Control leaf vs. GSNO leaf	1,548	1,555	59	166	1,271
Control leaf vs. control root	8,240	8,274	255	602	6,838
GSH leaf vs. GSH root	1,504	1,495	67	169	1,159
GSNO leaf vs. GSNO root	1,945	1,925	95	270	1,552

The results of the comparisons control root vs. GSH root were used to filter the results of GSNO root; and comparisons control leaf vs. GSH leaf to filter the results of GSNO leaf. Finally, GSNO leaf vs. GSNO root was filtered with comparisons of control leaf vs. control root and GSH leaf vs. GSH root.
RNA-seq analysis was carried out in root and leaf samples from 30-day-old Arabidopsis plants in which the roots were incubated for 3 h either with solutions of 1 mM GSNO (a NO donor) and 1 mM GSH or with distilled water as controls.
TSS, transcription start site.

1 mM GSH and distilled water (used as control). The comparison was: (i) control leaf vs. GSH leaf; (ii) control leaf vs. GSNO leaf; (iii) control root vs. GSH root; (iv) control root vs. GSNO root; (v) control leaf vs. control root; (vi) GSH leaf vs. GSH root; and (vii) GSNO leaf vs. GSNO root. The results found after the GSH and distilled water treatment (control) were used to filter the GSNO results (**Table 2**). The GSH treatment is important because it can mediate *S*-glutathionylation, the reversible formation of disulfide bridges between glutathione and cysteine residues, which has emerged as a possible PTM in several physiological or stress situations (Dalle-Donne et al. 2007).

To compare the effect of different treatments between both organs, we analyzed groups of genes for which the fold change (Fc) was $\geq$2 up and down for up-regulated and down-regulated

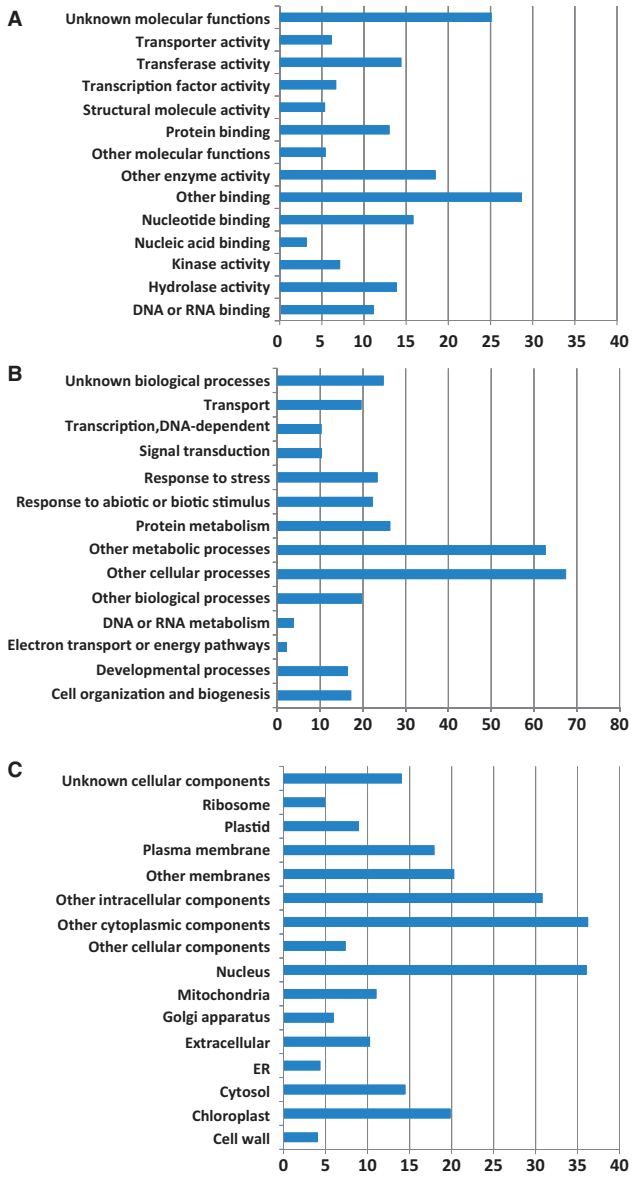


Fig. 2 Distribution of GSNO-responsive genes from whole Arabidopsis plants in several gene ontology (GO) categories. Genes with putative functions were assigned to (A) molecular function, (B) biological process or (C) cellular component categories using GO annotations from the TAIR database.

genes, respectively. **Fig. 3** shows volcano plots of significant genes plotted after RNA-seq analysis in the comparison of treatments (see **Table 2**) between Arabidopsis leaves and roots.

Transcriptomic analysis of GSNO-responsive genes in Arabidopsis roots

GSNO-responsive genes in Arabidopsis roots were analyzed, comparing the gene expression profile of two groups of 30-day-old plants, one treated with distilled water and the other one with 1 mM GSNO applied to the roots (control root vs. GSNO root). This result was filtered with GSH-responsive genes (comparison control root vs. GSH root) to eliminate genes that respond to GSH. The RNA-seq analysis showed that GSNO caused significant changes ($P < 0.005$) in gene expression levels of 1,715 genes in Arabidopsis roots. The functional classification of GSNO-induced genes (**Fig. 4**) shows that their products are predicted to be involved in the response to stress, biotic or abiotic stimulus, and protein metabolism processes. Furthermore, they have mainly nucleotide or protein binding and transferase or hydrolase activity. In addition, they are localized mostly in the nucleus, plasma membrane and chloroplast. On the other hand, the most abundant categories for GSNO-repressed genes in Arabidopsis roots (**Fig. 4**) are related to protein metabolism and they have a similar distribution in other categories such as developmental processes or stress response. These genes also have transferase, hydrolase, nucleotide and protein binding activities and they are localized mainly in the nucleus and plasma membrane. Within this group, we focused on 300 genes that showed greater response to GSNO, where 199 genes are up-regulated (Fc $\geq$2 up) and 101 genes down-regulated (Fc $\geq$2 down) 2-fold, respectively (**Supplementary Table S1**).

The importance of *S*-nitrosothiols has recently been demonstrated in the plant response both against pathogens (Feechan et al. 2005, Rusterucci et al. 2007, Chaki et al. 2009a) and to several abiotic stress situations in different plant species (Valderrama et al. 2007, Corpas et al. 2008, Chaki et al. 2011a, Chaki et al. 2011b; Airaki et al. 2011b). In this regard, RNA-seq analysis showed that GSNO provoked overexpression of disease resistance (*At3g04210*) or pathogenesis-related (*At4g33730*) proteins and five members of *WRKY* transcription factors (*At5g24110*, *At4g11070*, *At4g01250*, *At2g40740* and *At5g22570*), which constitute a family of genes that may be involved in the response to pathogens and abiotic stress (Rushton et al. 2010). Furthermore, GSNO caused the up-regulation of different cysteine-rich receptor-like protein kinases (*At4g11480* and *At4g23180*), which are a subgroup within the receptor like protein (RLP) family. In addition, GSNO caused expression changes of different *MYB* (myeloblast) family transcription factors (*At4g28110*, *At1g13300*, *At1g68670* and *At3g04030*).

Furthermore, different genes related to abiotic stress also respond to GSNO treatment such as heat stress transcription factors (*At3g63350*, Fc = 2.01 up) or wound-responsive proteins (*At5g58750* and *At4g10270* with Fc =2.70 up and 2.3 down, respectively). Recently, it has been reported that the wound-related protein *At5g58750*, which is up-regulated by GSNO, undergoes tyrosine nitration during natural senescence of pea plant roots (Begara-Morales et al. 2013a). Treatment of Arabidopsis plants with GSNO led to differential expression of genes related to oxidative stress, suggesting the relationship between NO and oxidative metabolism. In fact, alternative oxidase 1a has been reported to be induced in response to NO (Huang et al. 2002). In agreement with this, mitochondrial alternative oxidase 3 (*At1g32350*), peroxidase 7 (*At1g30870*), mitochondrial alternative oxidase 1a (*At3g22730*), peroxidase

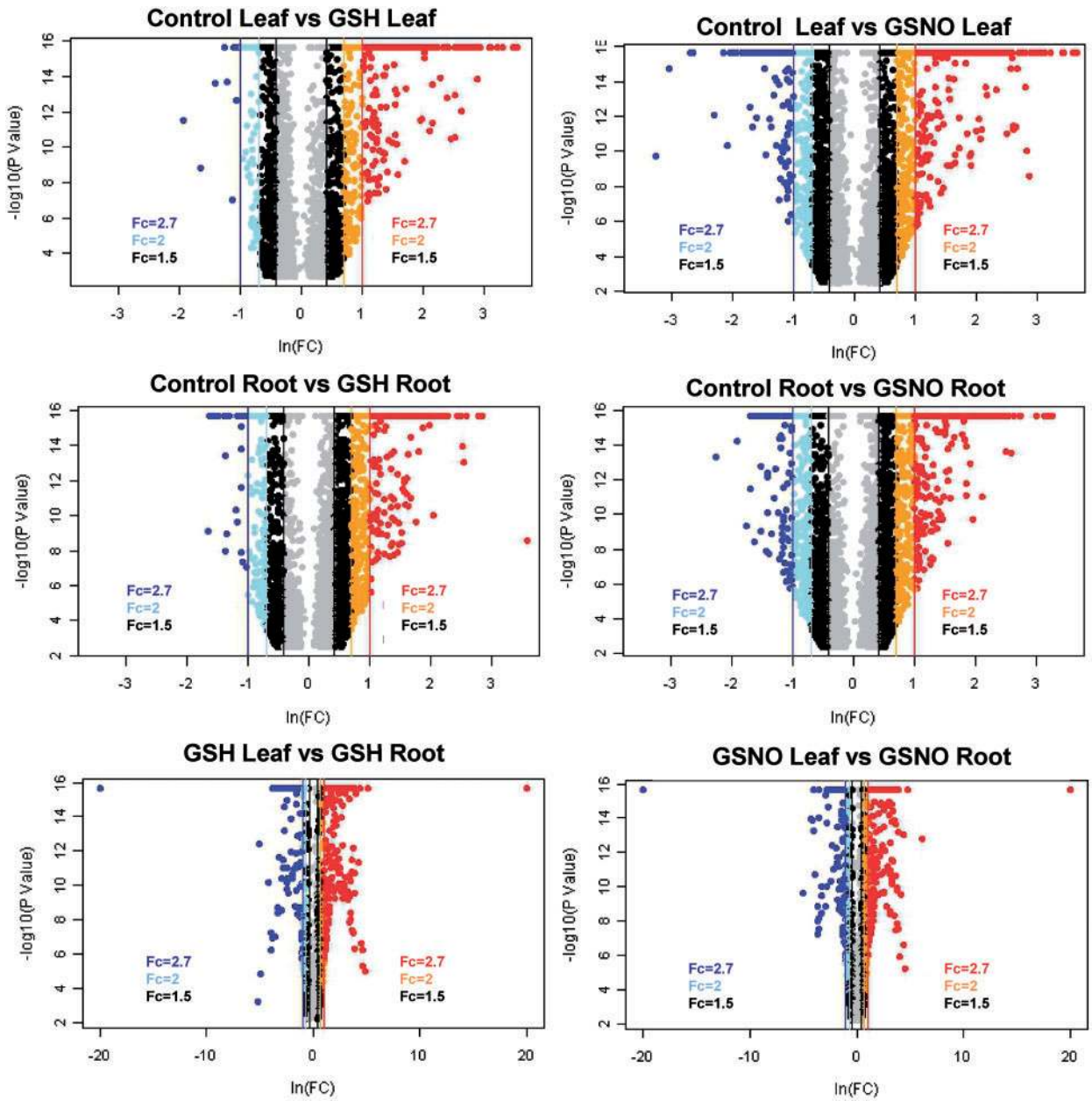


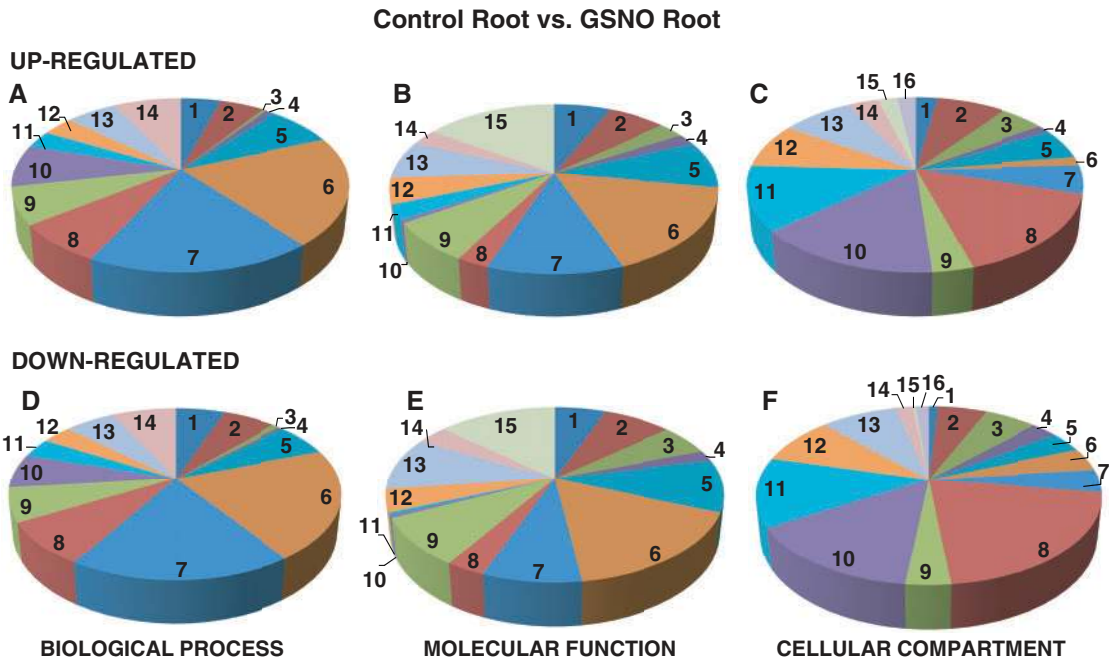
Fig. 3 Volcano plots of significant genes in leaf and root of Arabidopsis plants after RNA-seq analysis. The *x*-axis represents the natural logarithm of fold change (Fc) and the *y*-axis represents $\log_{10}$ of the *P*-value of each gene. Several breakpoints of the Fc values are indicated on the *x*-axis, where 0 indicates 'no change'. Up-regulated genes are shown in red (Fc = 2.7 up) and orange (Fc = 2 up), while down-regulated genes are shown in dark blue (Fc = 2.7 down) and light blue (Fc = 2 down). Genes with a slight change in expression are shown in black and gray.

35 (*At3g49960*), peroxidase 73 (*At5g67400*) and glutathione transferase TAU 8 (*At3g09270*) responded to GSNO.

Among the GSNO-responsive genes in roots, there are different genes called miscellaneous RNA (*miscRNA*) genes with Fc between 2.01 and 228.03 for up-regulated *miscRNA* genes and 1.59 and 2.27 for down-regulated genes. RNA-seq analysis also showed an increase due to GSNO in Arabidopsis roots in the transcript levels of chloroplastic glucose-6-phosphate-1-dehydrogenase 3 (G6PDH 3; *At1g24280*) and ferredoxin-NADP reductase (*FNR*) isozyme 2 (*At1g30510*).

Transcriptomic analysis of GSNO-responsive genes in Arabidopsis leaves

Because GSNO treatment was applied to the roots (**Fig. 1**), the analysis of gene expression changes in leaves helps us to understand the GSNO signaling mechanism in Arabidopsis plants. Therefore, GSNO-responsive genes in leaves were also analyzed, comparing the gene expression profile of control leaf vs. GSNO leaf. This result was filtered with GSH-responsive genes (comparison control leaf vs. GSH leaf) to ensure that the observed



#	GO Term	A	D
1	Cell organization and biogenesis	4.6	5.7
2	Developmental processes	5.1	6.3
3	DNA or RNA metabolism	0.5	1.0
4	Electron transport or energy pathways	1.1	0.5
5	Other biological processes	7.6	6.2
6	Other cellular processes	20.1	20.7
7	Other metabolic processes	18.5	18.5
8	Protein metabolism	7.5	8.2
9	Response to abiotic or biotic stimulus	6.8	6.3
10	Response to stress	8.0	6.5
11	signal transduction	3.1	3.6
12	Transcription.DNA-dependent	3.5	3.4
13	Transport	5.7	5.7
14	Unknown biological processes	7.8	7.5

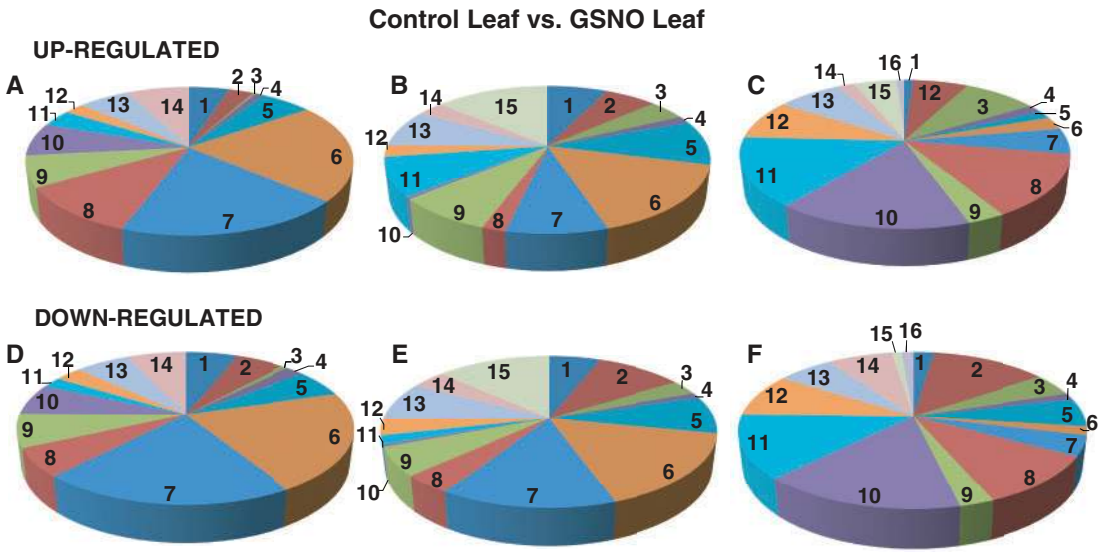
#	GO Term	B	E
1	DNA or RNA binding	6.7	5.9
2	Hydrolase activity	6.9	7.9
3	Knase activity	3.0	5.7
4	Nucleic acid binding	2.2	2.0
5	Nucleotide binding	8.9	9.6
6	Other binding	16.6	16.7
7	Other enzyme activity	11.2	8.0
8	Other molecular functions	2.7	3.2
9	Protein binding	7.5	8.9
10	Receptor binding or activity	0.6	0.5
11	Structural molecule activity	2.9	0.5
12	Transcription factor activity	5.0	4.2
13	Transferase activity	7.2	9.8
14	Transporter activity	3.1	3.5
15	Unknown molecular functions	15.5	13.6

#	GO Term	C	F
1	Cell wall	2.5	1.0
2	Chloroplast	7.9	5.5
3	Cytosol	5.3	6.0
4	Endoplasmic reticulum	1.5	2.9
5	Extracellular	5.6	3.7
6	Golgi apparatus	1.6	4.2
7	Mitochondria	5.2	4.0
8	Nucleus	15.8	21.0
9	Other cellular components	3.3	3.5
10	Other cytoplasmic components	15.0	14.7
11	Other intracellular components	12.1	12.6
12	Other membranes	8.1	8.5
13	Plasma membrane	8.1	8.6
14	Plastid	3.6	2.0
15	Ribosome	2.2	0.3
16	Unknown cellular components	2.2	1.5

Fig. 4 Functional classification of GSNO-responsive genes in roots of Arabidopsis plants. Genes were classified by functional categories under the following gene ontology (GO) terms: biological process (A, D), molecular function (B, E), and cellular compartment (C, F) and by up-regulated (A–C) or down-regulated (D–F) genes. The number of genes assigned to each functional category is expressed as a percentage (%).

gene expression changes were due to GSNO effects. RNA-seq analysis showed 1,548 genes significantly affected by GSNO treatment ($P < 0.005$) in leaves. The functional classification and distribution of GSNO-responsive genes in Arabidopsis leaves are shown in **Fig. 5**. For GSNO-induced genes, the most abundant categories correspond to genes involved in protein metabolism, response to stress and abiotic or biotic stimulus, and they have mostly binding or transferase activities. Furthermore, these genes are localized mainly in the nucleus, cytosol and plasma membrane. Within this group, we focused on genes that showed a greater response to GSNO, with 73 genes up-regulated and 76 genes down-regulated at least 2-fold (Fc $\geq$2 up and down) (**Supplementary Table S2**).

As in roots, GSNO induces the expression of different transcription factors belonging to *WRKY* (*At1g66600* and *At5g01900*) or *MYB* (*At1g66390* and *At1g06180*) family genes



BIOLOGICAL PROCESS

#	GO Term	A	D
1	Cell organization and biogenesis	4.8	6.0
2	Developmental processes	2.8	5.7
3	DNA or RNA metabolism	0.4	0.8
4	Electron transport or energy pathways	1.0	2.1
5	Other biological processes	6.0	5.9
6	Other cellular processes	21.4	20.9
7	Other metabolic processes	19.2	20.7
8	Protein metabolism	10.9	6.0
9	Response to abiotic or biotic stimulus	6.7	7.1
10	Response to stress	7.2	6.6
11	Signal transduction	3.7	2.2
12	Transcription.DNA-dependent	2.3	3.1
13	Transport	6.1	6.1
14	Unknown biological processes	7.5	6.9

MOLECULAR FUNCTION

#	GO Term	B	E
1	DNA or RNA binding	7.1	5.9
2	Hydrolase activity	6.2	10.2
3	Kinase activity	4.4	3.2
4	Nucleic acid binding	1.6	1.1
5	Nucleotide binding	9.7	7.9
6	Other binding	15.9	16.3
7	Other enzyme activity	8.6	14.3
8	Other molecular functions	2.0	4.1
9	Protein binding	8.1	6.0
10	Receptor binding or activity	0.5	0.6
11	Structural molecule activity	8.6	1.8
12	Transcription factor activity	2.6	3.4
13	Transferase activity	7.9	8.2
14	Transporter activity	3.2	4.0
15	Unknown molecular functions	13.6	13.1

CELLULAR COMPARTMENT

#	GO Term	C	F
1	Cell wall	1.0	2.3
2	Chloroplast	6.4	13.0
3	Cytosol	8.7	4.7
4	Endoplasmic reticulum	1.8	1.4
5	Extracellular	1.6	5.8
6	Golgi apparatus	2.7	1.8
7	Mitochondria	5.6	4.2
8	Nucleus	13.7	10.7
9	Other cellular components	3.1	2.7
10	Other cytoplasmic components	16.1	15.8
11	Other intracellular components	15.0	13.2
12	Other membranes	8.6	8.8
13	Plasma membrane	7.4	6.1
14	Plastid	2.1	7.2
15	Ribosome	5.0	1.1
16	Unknown cellular components	1.1	1.3

Fig. 5 Functional classification of GSNO-responsive genes in leaves of Arabidopsis plants. Genes were classified by functional categories under the following gene ontology (GO) terms: biological process (A, D), molecular function (B, E), and cellular compartment (C, F) and by up-regulated (A–C) or down-regulated (D–F) genes. The number of genes assigned to each functional category is expressed as a percentage (%).

and several genes related to biotic or abiotic stress response such as disease resistance (*At1g17610*, *At3g14470* and *At3g44400*) or RLP proteins (*At4g04540*, *At1g47890*, *At3g24900*, *At1g74170*, *At3g28890*, *At1g34420* and *At3g05660*).

RNA-seq analysis showed the identification of several genes involved in the metabolism of methionine sulfoxides (MetSOs) which alter both the activity and the conformation for many proteins. Specifically, methionine sulfoxide reductase (MSR) catalyzes the reduction of MetSO back to methionine and is considered a regulator of antioxidant defense. Thus, RNA-seq analysis showed that most of the *MSR* genes respond to GSH but not to GSNO in Arabidopsis leaves (**Table 3**). Furthermore, only the *MSRB7* (*At4g21830*) gene responded to GSNO and not to GSH under our experimental conditions (Fc $\geq$2).

Table 3 Fold changes of methionine sulfoxide reductase (*MSR*) genes from *Arabidopsis thaliana* in the different comparisons analyzed by RNA-seq

Name	Gene	Control leaf vs. GSNO leaf	Control leaf vs. GSH leaf	Control root vs. GSNO root	Control root vs. GSH root	GSNO leaf vs. GSNO root	GSH leaf vs. GSH root	Control leaf vs. control root
AtMSRA1	At5g61640	–	–	–	–	–	–	–
AtMSRA2	At5g07460	–	–	–	–	–	–	6.00↑
AtMSRA3	At5g07470	–	1.22↓	–	–	–	–	1.35↓
AtMSRA4	At4g25130	–	1.22↓	–	–	–	–	3.33↓
AtMSRA5	At2g18030	–	–	–	–	–	–	–
AtMSRB1	At1g53670	–	1.3↓	–	–	–	–	2.77↓
AtMSRB2	At4g21860	–	1.22↓	–	–	–	–	4.54↓
AtMSRB3	At4g04800	1.44↑	–	–	–	–	–	–
AtMSRB4	At4g04810	–	–	–	–	–	–	–
AtMSRB5	At4g04830	–	2.27↓	–	–	2.72↑	–	–
AtMSRB6	At4g04840	–	–	–	–	–	–	–
AtMSRB7	**At4g21830**	**7.85↑**	–	–	–	–	–	**74.9↑**
AtMSRB8	At4g21840	–	3.29↑	–	–	–	–	6.29↑
AtMSRB9	At4g21850	–	3.64↑	–	–	–	–	–

Most of these genes respond to GSH treatment, and only *MSRB7* (in bold) responds to GSNO and not to GSH under our experimental conditions (Fc ≥2 up and down, $P < 0.005$).

RNA-seq analysis was carried out in root and leaf samples of 30-day-old Arabidopsis plants in which the roots were incubated for 3 h either with solutions of 1 mM GSNO (NO donor) and 1 mM GSH or with distilled water as controls.

↑, up-regulated genes. ↓, down-regulated genes. '–', not affected.

On the other hand, the functional classification and distribution of GSNO-repressed genes in Arabidopsis leaves are similar to those described for up-regulated genes (**Fig. 5**). However, GSNO-repressed genes also have hydrolase activity and are located mainly in the chloroplast and other plastids. Among GSNO-repressed genes, there is a broad diversity of genes related to a variety of cellular processes, such as leucine-rich repeat (LRR) proteins (*At2g15880*), sugar transporter *ERD6-like 17* (*At5g27350*), *β-galactosidase 4* gene (*At5g56870*) or senescence-associated genes (*SAG12*, *At5g45890*). In this process, NO is a negative regulator of leaf senescence, and its content decreases with the age of the plant (Corpas et al. 2004, Procházková and Wilhelmová 2011). Furthermore, the *ATMGL* (*Arabidopsis thaliana methionine gamma-lyase*) gene (*At1g64660*) was also down-regulated. It encodes a cytosolic enzyme that catalyzes the degradation of methionine into methanethiol, α-ketobutyrate and ammonia, participating in methionine homeostasis (TAIR database).

Differential transcriptomic analysis of GSNO-responsive genes between Arabidopsis roots and leaves

Until now, we have analyzed separately a list of genes that respond to GSNO in Arabidopsis leaves and roots. However, one of the most informative comparisons of this study is to determine genes expressed differentially or specifically in both organs in response to GSNO. For this reason, we made the comparison GSNO leaf vs. GSNO root, the results of which were filtered with those found by comparisons of control leaf vs. control root and GSH leaf vs. GSH root. Thus, we eliminated the group of genes that respond to GSH or genes which had different expression between the two organs *per se*. From this standpoint, genes up-regulated or down-regulated are genes that increased (Fc ≥2 up) or decreased (Fc ≥2 down) their expression in roots with respect to leaves. RNA-seq analysis thus enabled us to identify 1,945 genes that responded differently to GSNO in roots and leaves, where there were 509 genes up-regulated and 308 down-regulated in roots with respect to leaves (**Supplementary Table S3**). These differentially expressed genes belong to functional categories similar to those described for leaf and root separately (**Supplementary Fig. S1**).

Since the GSNO treatment was performed via the root, the maximum differential gene expression was achieved with a specific response in root or leaf through genes that are expressed in one organ but whose expression is not detected in the other organ in response to GSNO. Thus, within the comparison GSNO leaf vs. GSNO root, we also identified a total of 114 genes that presented a specific modulation in root (66 root-specific genes) or leaf (48 leaf-specific genes), suggesting an organ-specific modulation of gene expression by NO. These genes are listed in **Supplementary Table S3**. The functional classification of these genes (**Supplementary Fig. S2**) showed that the specific leaf genes were involved mainly in the response to different types of stress and abiotic or biotic stimulus, whereas specific root genes participated mostly in developmental processes, although they also acted in response to stress and other biological processes. Furthermore, both groups of genes were located in the nucleus and plasma membrane. However, a large percentage of specific leaf genes were located in the chloroplast, while specific root genes belonged to the extracellular category.

Validation of GSNO-responsive genes by quantitative real-time reverse transcription–PCR (qRT–PCR)

To validate RNA-seq results, we randomly assigned several GSNO-responsive genes in roots (a total of eight genes) and leaves (a total of eight genes) to conduct the expression analysis by qRT–PCR. **Fig. 6** shows the comparison between the qRT–PCR and RNA-seq analysis, showing that all the GSNO-responsive genes tested and previously identified by RNA-seq were confirmed by qRT–PCR. The results showed a positive correlation between the two approaches (with a correlation coefficient of 0.9), indicating that the RNA-seq expression analysis performed is highly reliable.

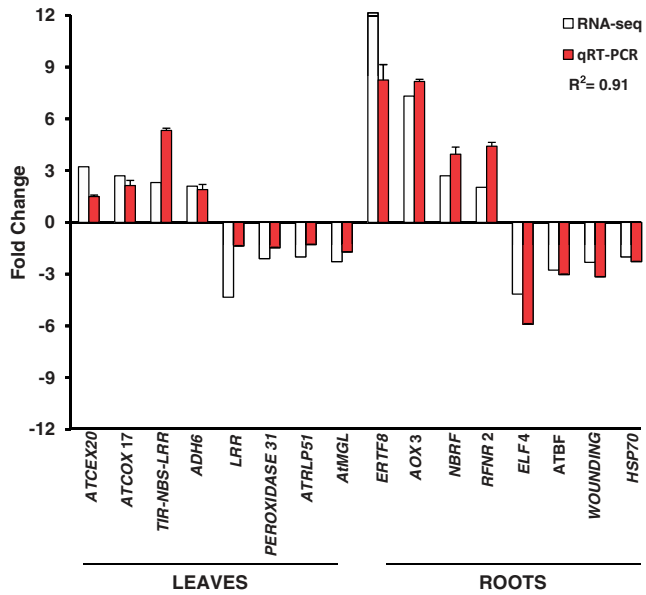


Fig. 6 qRT–PCR validation of RNA-seq results. Sixteen genes identified previously as GSNO-responsive genes by RNA-seq (white bar) in leaves and roots of Arabidopsis plants were randomly selected to analyze, by qRT–PCR, the differential expression changes (red bars). Comparison of fold change of RNA-seq and qRT–PCR shows a correlation coefficient of 0.91, indicating that RNA-seq results are reliable. Error bars represent the SEM. *ATCEX20* (AT5G62180), *Arabidopsis thaliana* carboxyesterase 20; *ATCOX 17* (AT3G15352), *Arabidopsis thaliana* cytochrome *c* oxidase 17; *TIR-NBS-LRR* (AT3G44400), disease resistance protein (TIR-NBS-LRR class); *ADH 6* (AT5G24760), alcohol dehydrogenase-like 6; *LRR* (AT2G15880), leucine-rich repeat family protein; *PEROXIDASE 31* (AT3G28200), putative peroxidase; *ATRLP51* (AT4G18760), receptor like protein 51; *AtMGL* (AT1G64660), methionine gamma-lyase; *ERTF8* (AT1G53170), ethylene-responsive transcription factor 8; *AOX 3* (AT1G32350), mitochondrial alternative oxidase 3; *NBRF* (AT5G58750), NAD(P)-binding Rossmann-fold superfamily protein; *RFNR 2* (AT1G30510), root ferredoxin NADP reductase; *ELF 4* (AT2G40080), early flowering 4; *ATBF* (AT1G49720), ABA-insensitve 5-like protein; *WOUNDING* (AT4G10270), wounding-responsive family protein; *HSP 70* (AT4G16660), heat-shock protein 70, putative.

Conserved motifs found on the promoters of GSNO-inducible genes

We found two statistically significant and possibly undiscovered core oligomers (AATTAT and AAAACA) on both root and leaf groups of genes and also by searching on those leaf and root gene promoters together. They were found to be present in 108 sequences out of 119 (binomial distribution *P*-value = 8.68e-04) and 117/119 (*P*-value = 2.34e-03), respectively. Either of these elements was present at least twice in 55% of promoters, with a maximum number of copies per strand of five for AATTAT and seven for AAAACA. The AATTAT copy nearest to the start codon was located within 1 and 300 bp upstream in 51% of the cases and within 1 and 500 bp upstream in 76% of the cases. Similarly, the AAAACA copy nearest to the start codon was located within −3 and −100 bp in 52% of the cases and within −3 and −400 bp in 80% of the promoters.

To find any known function for these elements, we used the referential plant transcription sites Plantcare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html). As a result, we found that these oligomers included each one in a different conserved motif (ACCAATTAT TGGTTACTAAATTT AACAG and ATTAAAAGTTAAAAACACA) but both were described as a GT-1 factor-binding site in *Phaseolus vulgaris* bean embryo. No conserved motifs similar to the selected oligomers were reported for Arabidopsis in Plantcare.

Discussion

GSNO belongs to a groups of NO-derived molecules designated as *S*-nitrosothiols which are involved in the mechanism of response against different adverse conditions such as pathogens (Feechan et al. 2005, Rusterucci et al. 2007, Chaki et al. 2009a) or abiotic stresses (Valderrama et al. 2007, Corpas et al. 2008, Airaki et al. 2011b, Chaki et al. 2011a, Chaki et al. 2011b, Leterrier et al. 2012). GSNO is formed by the interaction of NO with reduced glutathione (GSH) and it is considered an intracellular NO reservoir as well as a vehicle of NO throughout the cells. Therefore, in this study, GSNO was applied to the roots since the main goal of this work was to identify GSNO-responsive genes in the main organs (roots and leaves) of *A. thaliana* under non-stress conditions, using RNA-seq technology.

GSNO provokes expression changes of stress-related genes and organ-specific genes in both roots and leaves

Although GSNO has been previously used to analyze NO-responsive genes in plants (Ferrarini et al. 2008), GSH has not been used as an additional control. However, we believe that GSH treatment as an additional control was necessary in order to eliminate GSH-responsive genes that could trigger a signaling mechanism by *S*-glutathionylation that could interfere with the GSNO response. Bearing this in mind, we observed that GSNO

provokes overexpression of genes involved in disease resistance and pathogenesis both in roots and in leaves. These also include members of *WRKY* transcription factors, which constitute a family of genes that may be involved in the response to pathogens and abiotic stress (Rushton et al. 2010) as well as different cysteine-rich receptor-like protein kinases which are a subgroup within the RLP family. RLP proteins are cell surface receptors with an extracellular LRR domain that are involved in different processes, and some of them can participate in the response to pathogens (Wang et al. 2008, Wang and Fiers, 2010).

Furthermore, we observed that MYB transcription factors are targets of GSNO in both organs. In plants, *MYB* transcription factor genes are a large family functionally involved in the regulation of several plant-specific processes (Kirik et al. 1998, Stracke et al. 2001) and it has been reported that the interaction of *AtMYB2* transcription factor and DNA is inhibited by SNP and GSNO, through an *S*-nitrosylation mechanism (Serpa et al. 2007).

On the other hand, RNA-seq analysis showed that GSNO has a different effect in both organs. Thus, the functional analysis of organ-specific GSNO-responsive genes allowed us to determine that a major proportion of these genes encoded proteins involved in plant responses to several stress situations in leaves and developmental processes in roots, suggesting that NO signaling can be mediated by different responses, depending on the plant organ. These results open up a promising field in which future studies of these genes differentially or specifically expressed in both organs will enable us to delve into the NO signaling mechanisms under non-stress conditions in plants. As in the specific leaf genes, a high percentage of GSNO-responsive genes in the different treatments are also related to response to stress or biotic and abiotic stimulus. These results suggest that plants trigger the defense mechanisms against different adverse conditions in response to NO. In this regard, it has been shown that NO plays a key role in defense against biotic or abiotic stress situations (Delledonne et al. 1998, Durner et al. 1998, Corpas et al. 2011, Siddiqui et al. 2011). It has been reported that *S*-nitrosylation is an NO-dependent PTM involved mainly in defense mechanisms against abiotic or biotic stress conditions (Astier and Lindermayr 2012, Begara-Morales et al. 2014). Furthermore, Delledonne et al. (1998) reported that not only an oxidative burst, but also NO is necessary for the hypersensitive cell death in soybean cell cultures. These authors concluded that NO and reactive oxygen species (ROS) are complementary and trigger a synergistic induction of hypersensitive cell death. On the other hand, they showed that NO can activate genes involved in the hypersensitive response (HR) independently of ROS. Accordingly, it seems logical that RNA-seq analysis of Arabidopsis plants treated with GSNO showed that several signaling pathways involved in the defense mechanisms against different stress situations are triggered by NO. These results support the idea that NO acts as a signal molecule involved in the adaptive response to various plant stress situations (Besson-Bard et al. 2009). In agreement with this, recent new data clearly suggest that NO/GSNO/*S*-nitrosothiols can modulate ROS synthesis during the HR, in symbiotic interactions or under abiotic stress (Yu et al. 2012, Boscari et al. 2013, Corpas and Barroso, 2013, Scheler et al. 2013, Wang et al. 2013). Thus, there are several specific examples where *S*-nitrosylation is clearly involved, such as *S*-nitrosylation of NADPH oxidase, which inhibits the generation of superoxide radicals during HR (Yun et al. 2011), or *S*-nitrosylation of ascorbate peroxidase, which increases its activity and consequently allows the regulation of the hydrogen peroxidase content under salinity stress (Begara-Morales et al. 2014).

In roots, GSNO provokes overexpression of miscellaneous RNA and genes involved in the metabolism of NADPH

Among the GSNO-responsive genes identified in roots, there are different genes called miscellaneous RNA (*miscRNA*) genes. The *miscRNA* genes are a type of non-coding RNAs that do not encode proteins but that may be involved in several key biological processes (Amaral and Mattick 2008, Jamalkandi and Masoudi-Nejad 2009, Song et al. 2009). Our results suggest a regulation of *miscRNA* metabolism by NO, so that future studies are needed to elucidate the molecular function of these *miscRNA* genes and therefore the effect of NO in pathways or biological processes in which these genes are involved.

RNA-seq analysis also showed a rise in the transcript levels of G6PDH 3 which generates reducing power in the form of NADPH, an indispensable electron donor in several biosynthetic pathways and detoxification processes (del Río et al. 2002, Valderrama et al. 2006). It has been reported that the activity of a recombinant G6PDH from pea plants is progressively inhibited, depending on the GSNO concentration, while GSH causes no change (Begara-Morales 2011). This result suggests that G6PDH from pea plant is modified by GSNO through *S*-transnitrosylation. Therefore, the increase in *G6PDH* transcripts occurring after the treatment of Arabidopsis plants with GSNO could be a plant response to the loss of protein activity, in an attempt to maintain the levels of NADPH-generating protein de novo.

It is also worth noting that the ferredoxin-NADP reductase (*FNR*) isozyme 2 gene was also up-regulated by GSNO in Arabidopsis roots. FNR mediates the final step of photosynthetic electron flow by transferring electrons from ferredoxin to NADP with the concomitant generation of NADPH. However, in non-photosynthetic organs, FNR isoenzymes provide reduced ferredoxin for bioassimilation and biosynthesis enzymes (Wang et al. 2000, Hanke et al. 2005). The most abundant root isoform is AtFNR2, which is involved specifically in root nitrate assimilation (Wang et al. 2000, Hanke et al. 2005). Overexpression of root *FNR2* by GSNO suggests the involvement of NO in the nitrate uptake process in Arabidopsis plants. Recently, it has been shown that leaf FNR of pea plants has an affinity for GSNO and that FNR is also a target of tyrosine nitration in sunflower (Begara-Morales et al. 2013b, Chaki

et al. 2011b). Therefore, *FNR* is a good candidate to be modified by NO at the level both of gene expression and of protein PTMs.

In leaves, the *methionine sulfoxide reductase 7* (*MSR7*) gene is up-regulated by GSNO

Methionine oxidation by ROS or RNS (John et al. 2001, Alvarez and Radi 2003) leads to the formation of MetSO (Boschi-Muller et al. 2008) which could alter both the activity and the conformation of many proteins (Dos Santos et al. 2005, Rouhier et al. 2006, Hsu and Lee 2012). This oxidative damage is reversible because the enzyme MSR catalyzes the reduction of MetSO back to methionine. There are two structurally unrelated classes of MSRs called MSRA and MSRB, which catalyze the reduction of the MetSO '*S*' or '*R*' enantiomer to methionine, respectively (Rouhier et al. 2006, Boschi-Muller et al. 2008). MSRs have been related to plant response to oxidative stress (Dos Santos et al. 2005, Rouhier et al. 2006, Li et al. 2012) and to NO (Hsu and Lee 2012). In the present study, RNA-seq analysis showed that most of the *MSR* genes responded to GSH but not to GSNO in Arabidopsis leaves (**Table 3**). In contrast, only the *MSRB7* gene responded to GSNO and not to GSH under our experimental conditions (Fc $\geq$2).

MSRB7 was much more highly expressed in roots than in leaves (**Table 3**) because its transcripts are relatively abundant in roots (Rouhier et al. 2006), although there is expression in other organs including Arabidopsis leaves (Li et al. 2012). This result suggests that in leaves, where there is a very low expression of the *MSRB7* gene, it is necessary to increase its expression level to produce greater quantities of protein and thus respond to the possible methionine oxidation. However, *MSRB7* transcripts are abundant in roots where basal levels can be sufficient to reverse the possible oxidation of methionine generated under physiological or stress situations. In this regard, it has been shown that in Arabidopsis, exposure to 20 μM methyl viologen provoked an increase in *MSRB7* gene expression. Furthermore, Arabidopsis *msrB7* knockdown lines are sensitive to oxidative stress provoked by exposure to 20 μM methyl viologen or 20 mM hydrogen peroxide, whereas overexpression lines exhibit tolerance (Li et al. 2012).

MSR enzymes, including MSRB7, are repair systems that protect against methionine oxidation. However, GSNO cannot itself oxidize methionine, although it can give rise to NO, which may react with superoxide, leading to peroxynitrite (John et al. 2001), which is known to have the ability to oxidize methionine to MetSO (Alvarez and Radi 2003). In this regard, Chaki et al. (2011a, 2011b) reported that the increase in *S*-nitrosothiols can mediate a nitrosative stress by increasing the peroxynitrite and nitrotyrosine content under abiotic stress conditions. In this situation, *MSRB7* could protect against oxidative damage from RNS as reported by John et al (2001) for *Escherichia coli* and *Mycobacterium tuberculosis*. It has been suggested that denitration of proteins in *A. thaliana* could probably be mediated by a peptide methionine sulfoxide reductase (PMSR) under normal growth conditions since *pmsr2-1* mutants displayed elevated protein nitration at night (Bechtold et al. 2009). As mentioned above, GSNO induces an *MSR* gene which protects methionine against the oxidative process and at the same time GSNO repressed the *ATMGL* gene that degrades methionine, so that the metabolism of methionine appears to be important in Arabidopsis leaves in response to GSNO, as happens in *E. coli* (Flatley et al. 2005). Furthermore, the higher levels of *MSRB7* transcripts may be a response to a potential inactivation of the protein activity by GSNO through an *S*-transnitrosylation mechanism. No studies are as yet available on post-translational changes of MSR proteins by RNS, but it would be a good starting point for understanding their regulation by NO.

GT-1 factor-binding site is a conserved motif on the promoters of GSNO-inducible genes

In silico analysis of the promoter region of the genes affected by GSNO has allowed us to identify two core oligomers (AATTAT and AAAACA) which are both described as GT-1 factor-binding sites in *P. vulgaris* bean embryo. GT-1 is a transcription factor which can bind to one of the *cis*-acting elements as BoxII (the core recognition sequence is GGTTAA) which is located in the upstream promoter region of light-responsive genes (Hiratsuka et al. 1994, Zhou 1999). In addition, there is experimental evidence that GT-1 may act as a molecular switch which is modulated by calcium-dependent phosphorylation and dephosphorylation in response to light stimuli (Maréchal et al. 1999, Nagata et al. 2010). On the other hand, it is worth noting that, among the GSNO-inducible gene promoters, there are two promoters with seven copies of AAAACA, one being the *At5g13220* promoter. The *At5g13220* unigene is induced by GSNO in leaves and it is described in TAIR as JAZ 10, which is a negative regulator of jasmonic acid (JA) signaling and disease symptom development (Demiansky et al. 2012). The other AAAACA-enriched promoter is that of *At1g67328*, a potential natural antisense gene of still unknown function that is induced by GSNO in roots.

In the case of AATTAT, there are also two promoters with five copies of this element. One of these promoters belongs to *At1g35560*, which is overexpressed in GSNO-treated roots and encodes a sequence-specific DNA-binding transcription factor involved in controlling flowering time and plant development (Balsemão-Pires et al. 2013). The other promoter corresponds to *At3g49570*, which encodes LSU3, a receptor-like kinase gene (RKL, subfamilly LRR XI) that has been observed to be down-regulated in response to glucose (Chae et al. 2009) and down-regulated as part of the basal response to phosphate starvation, specifically in roots (Woo et al. 2012). Now we report, also specifically in roots, LSU3 up-regulation in response to GSNO.

Overall, the most remarkable fact is that the majority of the genes induced by GSNO share these elements on the promoters. In addition, the genes with a higher copy number of any of these elements code for proteins that regulate different general upstream signaling pathways. Further research on these

motifs could extend our knowledge of GSNO as a signaling molecule.

In summary, in recent years, new high-throughput sequencing methods termed RNA-seq have emerged as useful tools that can expand the results achieved with array-based methods. Thus, using RNA-seq technology developed by Illumina, we identified a wide diversity of GSNO-responsive genes in the roots and leaves of Arabidopsis plants. Although most of these genes, which are involved in the protection mechanisms against several stress situations, have been described previously, we have identified target genes of GSNO which had not been identified by array-based methods, such as *MSR* genes in leaves or different *miscRNA* genes in roots of Arabidopsis plants. Furthermore, we have identified 1,945 genes that respond differently to GSNO in leaves and roots, and 114 genes that have organ-specific responses to NO, suggesting that NO signaling mechanisms can be mediated by different responses, depending on the plant organ in Arabidopsis plants. These genes constitute a good candidate for understanding the signaling mechanisms mediated by GSNO and for probing deeper into specific gene modulation by NO in different organs under unstressed conditions in plants.

Materials and Methods

Plant material, growth conditions and treatments

Arabidopsis thaliana ecotype Columbia seeds were surface-sterilized for 5 min in 70% ethanol containing 0.1% SDS, then for 20 min in sterile water containing 20% bleach and 0.1% SDS, washed four times in sterile water and germinated in vermiculite/sand (ratio 1/2) for 14 d. Healthy and vigorous seedlings were selected and grown in hydroponic culture for 16 d with a basal nutrient solution (Cellier et al. 2004). The environmental growth chamber parameters were as follows: light/dark cycle 8/16 h, light intensity 190 μE m^{-2} s^{-1}, temperature 22°C/18°C, 70% relative humidity. The nutrient solution was renewed once weekly during the first part of the culture, and daily in the last week before the experiment and during the experiment. For the experiments with GSNO, in 30-day-old plants, the nutrient solution was removed, root systems were washed with distilled water and then incubated for 3 h either with solutions of 1 mM GSNO (NO donor) and 1 mM GSH or with distilled water as controls. Thus, the treatments were performed under non-stress conditions (**Fig. 1**). Then, leaves and roots from each treatment were harvested and used for RNA isolation. Samples were designated as control leaf and root, GSH leaf and root, and GSNO leaf and root. A total of eight independent and healthy plants were used for these experiments.

RNA isolation and paired-end mRNA library preparation and sequence generation

Total RNA from pooled leaf or root organs was isolated using Trizol Reagent (Gibco-BRL) as described in the manufacturer's manual. Total RNA quality and concentration were determined using a NanoDrop 1000 Spectrophotometer and an RNA Nano Chip on a Bioanalyzer (Agilent). The enrichment of poly(A)$^+$ RNA was performed from 8 μg of total RNA using magnetic beads with oligo(dT). Then, the purified mRNA fragmentation was performed by incubation with heat, and the fragmented RNA was precipitated at −80°C with sodium acetate (NaOAc), glycogen and ethanol. The first-strand synthesis of cDNA was performed using random primers and enzyme SuperScript II, while the second strand was synthesized using DNA polymerase I. Subsequently, cDNA purification was performed using the QIAquick PCR Purification kit (Qiagen) and the ends were repaired using T4 DNA polymerase, Klenow DNA polymerase and T4 PNK. The cDNA was purified (QIAquick PCR Purification kit; Qiagen) and a Klenow (-exo) was used to add an adenine nucleotide to the 3′ ends. Samples were purified with the MinElute PCR Purification kit (Qiagen), and specific adaptors of the Illumina platform and paired-end protocol were ligated to both ends using T4 DNA ligase. The favorable sequencing fragments (400 bp) were purified by agarose gel using the QIAquick Gel Extraction kit (Qiagen), and library amplification was carried out with 15 cycles of PCR (Phusion DNA polymerase). Prior to cluster generation (Paired-end Cluster Generation kit v4), library quality and concentrations were determined using a DNA 1000 Chip on a Bioanalyzer (Agilent).

Libraries were sequenced on a Genome Analyzer II module using a 36-Cycle Sequencing kit v4.0 which allows sequence reads of 105 bases from both ends. The Genome Analyzer Sequencing Control v2.8 was used as control software. The sequences generated on the Genome Analyzer II were analyzed with the Genome Analyzer SCS/RTA + OLB 1.8 software. The Bustard algorithm was used to transform the detected intensity signals at the Genome Analyzer into DNA sequence, and a quality control was conducted using FastQC 0.8 software and Phred Score measure (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). Generated sequences were aligned against the TAIR database using TopHat (Trapnell et al. 2009) and Bowtie (Langmead et al. 2009) software, and the expression level of each predicted transcript (FPKM value) was estimated using Cufflinks (Trapnell et al. 2010).

Data analyses

Bioinformatic approaches were used to establish gene expression changes. First, we set an arbitrary threshold of 80 FPKM, which was determined by comparing the expression of several housekeeping genes (GAPA-2, RPL 2 and ACT 12) in all the analyzed samples, and used these values as an internal expression control. Furthermore, to determine gene expression changes occurring as a result of the different treatments, we made the following comparisons: (i) control leaf vs. GSH leaf; (ii) control leaf vs. GSNO leaf; (iii) control root vs. GSH root; (iv) control root vs. GSNO root; (v) control leaf vs. control root; (vi) GSH leaf vs. GSH root; and (vii) GSNO leaf vs. GSNO root. Expression changes due to GSH (comparisons i, iii and vi) and

distilled water (comparison v) were used as controls and to filter the results of treatments with GSNO (comparisons ii, iv and vii).

For functional annotation analyses, genes showing significant expression level changes in response to different treatments and comparisons were analyzed using DAVID (Dennis et al. 2003, Huang et al. 2009) and TAIR (http://www.arabidopsis.org/tools/bulk/go/index.jsp) databases.

Quantitative real-time reverse transcriptase–PCR (qRT–PCR)

Total RNA from leaves and roots of Arabidopsis plants treated with GSH or GSNO was isolated as above, and first-strand cDNA was synthesized using the First Strand cDNA Synthesis kit (Roche) in a final volume of 20 µl according to the manufacturer's instructions. Real-time PCR was performed in a CFX96 real-time PCR Detection System (Bio-Rad). Amplifications were carried out in 10 µl of total volume containing 10 ng of cDNA, 2 µM of specific primers (see **Supplementary Table S4**) and SsoFast EvaGreen Supermix (Bio-Rad). PCR conditions used consisted of an initial denaturation at 95°C for 30 s, followed by 39 cycles at 95°C, 3 s and 60°C, 7 s. After cycling, melting curves of the reaction were run from 65°C to 95°C. Results were normalized using *Actin12* (AT3G46520) and *L2* (AT2G44065) as internal controls. Each PCR was performed at least three times, with three independent samples.

In silico conserved motif search on promoters

To facilitate the identification of GSNO regulatory motifs, we looked for conserved elements by using TAIR web Tools (http://www.arabidopsis.org/ tools/bulk/ sequences/index.jsp) on both strands of the promoter (defined as the 1,000 bp sequence upstream of the start codon) of those genes that showed a greater response (Fc >2.7) to GSNO, with 35 genes up-regulated in leaves and 84 genes up-regulated in roots.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by ERDF-co-financed grants from the Ministry of Science and Innovation [BIO2009-12003-C02-01, BIO2009-12003-C02-02 and BIO2012-33904] and Junta de Andalucía (group BIO286 and BIO192), Spain; the Ministry of Science and Innovation [a PhD fellowship (FPU) to J.B.M.].

Acknowledgments

Special thanks are given to Mr Carmelo Ruíz-Torres and Miss María J Campos-Ramos for their excellent technical support.

Disclosures

The authors have no conflicts of interest to declare.

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