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Differential transcriptomic analysis by RNA-seq of GSNO-responsive genes between Arabidopsis roots and leaves.

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Abbreviations - FPKM, Fragments Per Kilobase of exon per Million fragments Mapped; Fc, Fold change; GO, gene ontology; GSH, glutathione; GSNO, *S*-nitrosoglutathione; *miscRNA,* miscellaneous RNA; MSR, methionine sulfoxide reductase; NO, nitric oxide; NO_2-Tyr, 3-nitrotyrosine; PTM, post-translational modification; ROS, reactive oxygen species; RNS, reactive nitrogen species; SNP, sodium nitroprusside.

Abstract

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. Using 30-day-old Arabidopsis plants grown under hydroponic conditions, exogenous 1 mM GSNO was applied to root systems for 3 h. Differential gene-expression analyses were made both in roots and in leaves by RNA sequencing (RNA-seq). A total of 3.263 genes were identified to be modulated by GSNO. Most of the genes identified were associated with the protection mechanism against stress situations, many of these being previously identified as target genes of GSNO by array-based methods. However, new genes were identified such as methionine sulfoxide reductase (MSR) in leaves or different miscellaneous RNA (*miscRNA*) in roots of Arabidopsis plants. 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 Arabidopsis organs. In summary, it is demonstrated that RNA-seq extends our knowledge of GSNO as a signalling molecule which differentially modulates gene expression in roots and leaves under non-stress conditions.

Introduction

Nitric oxide (NO) has been reported to be involved in several physiological or stress-response processes in plants. Furthermore, there are 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, hundreds of proteins have been identified as NO targets in plants in recent years (Lindermayr and Durner, 2007; Chaki et al., 2009b,2012; Lozano-Juste et al., 2011; Astier et al., 2012; Kato et al., 2013; Mengel et al., 2013; Begara-Morales et al., 2013a,b). *S*-nitrosoglutathione (GSNO) is a major low-molecular weight *S*-nitrosothiol (SNO) that is considered to be an endogenous reservoir of nitric oxide (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 as a vehicle of NO for long distances, this being important for the redox signalling mechanisms (Malik et al., 2011). GSNO is decomposed to oxidized glutathione (GSSG) and NH_3 by a NADH-dependent *S*-nitrosoglutathione reductase (GSNOR), consequently, this enzyme can regulate the GSNO content under physiological and stress conditions (Feechan et al., 2005; Rusterucci et al., 2007; Lee et al., 2008; Leterrier et al., 2011; Xu et al., 2013; Corpas et al., 2013). In addition, GSNO can carry out transnitrosylation reactions in which a 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; Kato et al., 2013; Begara-Morales et al., 2013b,c). In addition, NO-signalling through protein-post-translational modifications (PTMs) and NO-dependent signalling mechanisms based on changes in gene expression are also part of the signalling pathway. There is evidence that NO acts as a signalling 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, analyzing 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 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 genes 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 a NO donor, while GSNO has been used only to analyse 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-amplification fragment-length polymorphism (AFLP) and microarray technology (for review, see Besson-Bard et al., 2009). However, in recent years, new high-throughput sequencing methods, termed massively parallel sequencing or RNA-seq has emerged as useful tool that could replace and improve existing methods because of its advantages over array-based methods (Wilhelm and Landry, 2009; Van Verk et al., 2013): 1) it is not necessary to have previous knowledge of the transcribed regions so that it is easier to make gene-expression studies in complex organisms; 2) 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 3) RNA-seq output is at the theoretical maximum of base-pair resolution. As a result, very recently RNAseq data has started to be obtained in higher plants (Lee et al., 2010; Donà et al., 2013; Van Moerkercke et al., 2013; de Cremer et al., 2013; Postnikova et al., 2013).

The aim of the present study is to analyse the differential expression of the GSNO-responsive genes between roots and leaves under no-stress conditions using Arabidopsis as the model plant. This large-scale gene-expression analysis has been performed using the Pair-end RNA-seq technology developed by Illumina, being to our knowledge the first available report in plants to use this approach in order to understand the GSNO-signalling mechanism between different organs in plants. It allowed us to identify genes and biological pathways that respond to GSNO-signalling which previously were not identified by array-based methods in plants.

Results and discussion

Figure 1A shows the appearance of 30-day-old Arabidopsis plants and the experimental design used to apply throughout the roots either solutions of 1mM GSNO (NO donor), 1mM GSH or distilled water (control). Figure 1B illustrates schematically the process flux of the experimental procedure. Thus, leaves and roots from each treatment were harvested and used for total RNA isolation. Then, Pair-end libraries were prepared and sequenced as described in 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 above 80% in all cases (20 units or more in Phred values which corresponds to a sequencing error rate of 1%).

The alignment vs. Arabidopsis sequence was performed using TopHat (Trapnell et al., 2009) and Bowtie (Langmead et al., 2009) software. Following this, we used Cufflinks (Trapnell et al., 2010) program, 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, transcription start sites (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 above 20 units, and the minimum 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 assume that a gene with a FPKM value >80 is expressed, while a FPKM value <80 indicates that the gene is not expressed.

GSNO-responsive genes in Arabidopsis plants

The treatment of roots of Arabidopsis plants with 1mM 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. 2A), 2,869 sequences were assigned to biological-process (Fig. 2B), and 2,927 sequences were assigned to cell-component (Fig. 2C).

To determine the potential GSNO-signalling mechanisms between roots and leaves, we also compared RNA-seq results found in these organs after different root treatments with 1 mM
GSH and distilled-water (used as control). The comparison was: 1) Control Leaf vs. GSH Leaf; 2) Control Leaf vs. GSNO Leaf; 3) Control Root vs. GSH Root; 4) Control Root vs. GSNO Root; 5) Control Leaf vs. Control Root; 6) GSH Leaf vs. GSH Root; and, 7) 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). 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). Although GSNO has been previously used to analyse NO-responsive genes in plants (Ferrarini et al., 2008), GSH has not been used as additional control. However, we believe that GSH treatment as additional control is necessary in order to eliminate GSH-responsive genes which could trigger a signalling mechanism by *S*-glutathionylation that could interfere with GSNO-response.
To compare the effect of different treatments between both organs, we analysed groups of genes for which the Fold Change (Fc) was ≥ 2 up and down for up-regulated and down-regulated genes, respectively. Figure 3 shows volcano plots of significant genes obtained after RNA-seq analysis in the comparison among treatment (see Table 2) between leaf and root of Arabidopsis plants.

Transcriptomic analysis of GSNO-responsive genes in Arabidopsis roots

GSNO-responsive genes in Arabidopsis roots were analysed, comparing the gene-expression profile of two groups of 30-day-old plants, one treated with distilled water and the other one with 1mM GSNO throughout 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 Arabidopsi*s* 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 respond to GSNO, where 199 genes are up-regulated (Fc ≥ 2 up) and 101 genes down-regulated (Fc ≥ 2 down) two fold, respectively (Supplementary Table S1).

The importance of *S*-nitrosothiols has recently been demonstrated both in the plant response 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., 2010,2011; Airaki et al., 2011b). In this regard, RNA-seq analysis showed that GSNO provoked over-expression of disease resistance (*At3g04210*) or pathogenesis-related (*At4g33730*) proteins and five members of *WRKY* transcription factors (*At5g24110, At4g11070, At4g01250, At2g40740*, and *At5g22570*), which are 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 kinase (*At4g11480* and *At4g23180*), which are a subgroup within Receptor Like Proteins (RLP) family. RLP proteins are cell-surface receptors with an extracellular leucine-rich repeat 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). In addition, GSNO caused expression changes of different *MYB* (Myeloblast) transcription factors family (*At4g28110*, *At1g13300*, *At1g68670* and *At3g04030*). In this sense, 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). In plants, *MYB* genes are a large family functionally involved in the regulation of several plant-specific processes (Kirik et al., 1998; Stracke et al., 2001). 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 this sense, mitochondrial alternative oxidase 3 (*At1g32350*), peroxidase 7 (*At1g30870*), mitochondrial alternative oxidase 1a (*At3g22730*), peroxidase 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 (*miscRNAs*) with Fc between 2.01 and 228.03 for up-regulated *miscRNAs* and 1.59 and 2.27 for down-regulated ones. The *miscRNAs* are a type of non-coding RNAs (*ncRNA*) that do not encode proteins but they 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 *miscRNAs* 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 chloroplastic glucose-6-phosphate-1-dehydrogenase 3 (*At1g24280*) 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 cytosolic recombinant G6PDH from pea plants is progressively inhibited, depending on GSNO concentration, while GSH do not cause any 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 also bears noting that the ferredoxin-NADP reductase (*FNR*) isozyme 2 (*At1g30510*) gene is up-regulated by GSNO in Arabidopsis roots. In non-photosynthetic organs, FNR isoenzymes provide reduced Fd 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). Over-expression 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 presents an affinity for GSNO and that FNR is also a target of tyrosine nitration in sunflower (Begara-Morales et al. 2013b; Chaki et al., 2011).

Therefore, *FNR* is a good candidate to be modified by NO at both levels gene expression and protein post-translational modifications.

Transcriptomic analysis of GSNO-responsive genes in Arabidopsis leaves

Because GSNO treatment was performed throughout the roots (Fig. 1), the analysis of gene-expression changes in leaves helps us to understand the GSNO-signalling 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 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 greater respond to GSNO, with 73 genes up-regulated and 76 genes down-regulated at least twofold ($Fc \geq 2$ 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 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*).

Methionine oxidation by ROS or RNS (John et al., 2001; Alvarez and Radi, 2003) leads to the formation of methionine sulfoxides (MetSO) (Boschi-Muller et al., 2008) which could alter both the activity and the conformation for many proteins (Dos Santos et al., 2005; Rouhier et al., 2006; Hsu and Lee, 2012). This oxidative damage is reversible because the enzyme methionine sulfoxide reductase (*MSR*) catalyses the reduction of MetSO back to methionine. There are two structurally unrelated classes of *MSRs* called *MSRA* and *MSRB*, which catalyse the reduction of 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 *MSRs* genes respond to GSH but not to GSNO in Arabidopsis leaves (Table 3). Furthermore, only *MSRB7* (*At4g21830*) gene responded to GSNO and not to GSH under our experimental conditions (Fc ≥ 2).

MSRB7 is much more expressed in roots than in leaves (Fc=74.9 up) (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 *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 sense, it has been shown that Arabidopsis plant exposed to 20 µM methyl viologen provoked an increase of the *MSRB7* gene expression. Furthermore, Arabidopsis *msrB7* knockdown lines are sensitive to oxidative stress provoked by exposition 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 oxidation of methionine. However, GSNO cannot itself oxidize methionine, although it can give a rise to NO, which may react with superoxide, leading to peroxynitrite (John et al., 2001), which is known to have the ability to oxidize Met to Met-SO (Alvarez and Radi, 2003). In this sense, Chaki et al. (2010, 2011) reported that the increase of *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 *E. coli* and *M. tuberculosis.* It has been suggested that denitration of proteins in *A. thaliana* could be probably mediated by a peptide methionine sulfoxide reductase (PMSR) under normal growth conditions since *pmsr2-1* mutants displayed elevated protein nitration in the night (Bechtold et al., 2009). Furthermore, the higher levels of *MSRB7* transcripts may be a response to a potential inactivation of the protein activity by GSNO through a *S*-transnitrosylation mechanism. To our knowledge, post-translational changes of *MSR*s proteins by RNS have not yet been studied, but it would be a good starting point for understanding their regulation by NO.

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

localized 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 a senescence-associated genes (*SAG12, At5g45890*), a process in which 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, also down-regulated was the *ATMGL* (*Arabidopsis thaliana methionine gamma-lyase*) gene (*At1g64660*) that encodes a cytosolic enzyme which catalyses the degradation of methionine into methanethiol, α-ketobutyrate and ammonia, participating in the methionine homeostasis (*TAIR database*). As a mentioned above, GSNO induced a *MSR* gene which protect methionine against oxidative process and at the same time GSNO repressed a 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).

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

Until now, we have analysed separately a list of genes that respond to GSNO in Arabidopsis leaves and roots. However, one of the most informative comparisons of this study was 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 group of genes that respond to GSH or genes which had different expression between both 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. In this sense, RNA-seq analysis 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 similar functional categories as described for leaf and root separately (Supplementary Figure S1).

Since the GSNO treatment was performed by 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 the expression is not detected in the other one in response

to GSNO. In this sense, within the comparison GSNO Leaf vs. GSNO Root, we also identified a total of 114 genes that presented a specific modulation in root (66 genes root-specific) or leaf (48 genes leaf-specific), 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 Figure 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 chloroplast, while specific root genes belonged to the extracellular category. Taking these results together, we found through RNA-seq and functional analysis of organ-specific GSNO-responsive genes that a major proportion of these genes encoded proteins involved in plant response to several stress situations in leaves and developmental processes in roots, suggesting that NO signalling can be mediated by different responses, depending on the plant organ. These results open a promising field in which future studies of these genes differentially or specifically expressed in both organs will allow us to delve into the NO-signalling mechanisms under no-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 defence mechanisms against different adverse conditions in response to NO. In this regard, it has been shown that NO plays a key role 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 a NO-dependent post-translational modification involved mainly in defence mechanisms against abiotic or biotic stress conditions (Astier and Lindermayr, 2012; Begara-Morales et al., 2013c). 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 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 hypersensitive response independently of ROS. Accordingly, it seems logical that RNA-seq analysis of Arabidopsis plants treated with GSNO showed that several signalling pathways involved in the defence 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 this sense, recent new data clearly suggest that NO/GSNO/*S*-nitrosothiols can modulate reactive oxygen species (ROS) synthesis during hypersensitive response (HR), symbiotic interactions or under abiotic stress (Yu et al., 2012; Wang et al., 2013; Scheler et al., 2013; Boscari et al., 2013; Corpas and Barroso, 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 radical during HR (Yun et al., 2011) or *S*-nitrosylation of ascorbate peroxidase which increases its activity and consequently it allows to regulate the hydrogen peroxidase content under salinity stress (Begara-Morales et al., 2013c).

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

In order to validate RNA-seq results, several GSNO-responsive genes were randomly selected in roots (a total of 8 genes) and leaves (a total of 8 genes) to carry out expression analysis by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Figure 6 shows the comparison between 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. Results showed a positive correlation between both approaches (with a correlation coefficient of 0.9) indicating that the RNA-seq expression analysis performed is highly reliable.

Conserved motifs find on the promoters of GSNO inducible genes

We found two statistically significant and possibly uncovered 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. Any of these elements was present at least 2 times in 55% of promoters with a maximum number of copies for strand of 5 for AATTAT and 7 copies for AAAACA. The AATTAT copy nearest to the start codon was located within 1 and 300 upstream in 51% of cases and within 1 and 500 upstream in 76% of cases. Similarly, the AAAACA copy nearest to the start codon was located within -3 and -100 in 52% of the cases and within -3 and -400 in 80% of promoters.

In order to find any known function for these elements, the referential plant transcription sites Plantcare database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) was used. As a result, we

found these oligomers included each one in a different conserved motif (ACCAATTAT TGGTTACTAAATTTAACAG and ATTAAAAGTTAAAAACACA) but both described as GT-1 factor binding site in *Phaseolus vulgaris* bean embryo. No conserved motifs similar to selected oligomers were reported for Arabidopsis in Plantcare. 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 evidences 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 interesting to note that, among the GSNO inducible gene promoters, there are two promoters with seven copies of AAAACA. One of them is *At5g13220* promoter. *At5g13220* unigene is induced by GSNO in leaves and it is described in TAIR as JAZ 10 which is a negative regulator of both jasmonic acid (JA) signalling 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 over-expressed in GSNO treated roots and encodes a sequence-specific DNA binding transcription factor involved in flowering time control 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 for 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 signalling pathways. Further research on these motifs could extend our knowledge of GSNO as a signalling 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 methionine sulfoxide reductase in leaves or different miscRNA 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-signalling mechanisms can be mediated by different responses depending on the plant organ in Arabidopsis plants. These genes constitute a good candidate for understanding the signalling mechanisms mediated by GSNO and for probing deeper into specific gene modulation by NO in different organs under no-stress conditions in plants.

Material 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, and washed four times in sterile water and germinated in vermiculite/sand (relation 1/2) for 14 days. Healthy and vigorous seedlings were selected and grown in hydroponic culture for 16 days 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 22ºC/18ºC, 70% hygrometry. The nutrient solution was renewed once weekly during the first part of the culture, and daily 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 throughout the root for 3 h either with solutions of 1mM GSNO (NO donor) and 1mM 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 manufacture's manual. Total RNA quality and concentration

were determined using a NanoDrop 1000 Spectrophotometer and a RNA Nano Chip on a Bioanalyzer (Agilent). The enrichment of polyA+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 SuperScrip II, while the second one was synthesized using DNA pol 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 adapters of Illumina platform and Pair-end protocol were ligated to both ends using T4 DNA Ligase. The favourable sequencing fragments (400 bp) were agarose-gel purified using 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), libraries quality and concentration 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 analysed 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 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 analysed samples, and using 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: (1) Control Leaf vs. GSH Leaf, (2) Control Leaf vs. GSNO Leaf, (3) Control Root vs. GSH Root, (4) Control Root vs. GSNO Root, (5) Control Leaf vs. Control Root, (6) GSH Leaf vs. GSH Root, and (7) GSNO Leaf vs. GSNO Root. Expression changes due to GSH (comparisons 1, 3, and 6) and distilled water (comparison 5), were used as controls and to filter the results of treatments with GSNO (comparisons 2, 4, and 7).

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

Quantitative real-time reverse transcriptase polymerase chain reaction (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 synthesised 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 95ºC for 30 s, followed by 39 cycles at 95 ºC, 3 s and 60 ºC, 7s. After cycling, melting-curves of the reaction were run from 65 ºC to 95ºC. Results were normalized using *Actin12* (AT3G46520) and *L2* (AT2G44065) as internal controls. Each PCR reaction was performed at least three times, with three independent samples.

***In silico* Conserved motifs search on promoters:**

In order to potentially 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 1000 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.

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1

2

Table 1. Total number and percentage in high quality of fragments obtained in both directions of sequencing by RNA-seq of roots and leaves samples of 30-day-old Arabidopsis plants incubated throughout the roots for 3 h either with solutions of 1mM GSNO (a NO donor) and 1mM GSH or with distilled water (control). Percentage of high quality is referred to Phred value which is widely accepted to characterize the quality of DNA sequences, and used to evaluate the efficacy of the sequencing method.

Sample	Total number 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

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 Cufflinks program. 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 filter with Control Leaf vs. Control Root and GSH Leaf vs. GSH Root comparisons. RNA-seq analysis was done in roots and leaves samples of 30-day-old Arabidopsis plants incubated throughout the roots for 3 h either with solutions of 1mM GSNO (a NO donor) and 1mM GSH or with distilled water as controls. TSS: transcription start site.

Comparison	Genes	Isoforms	Promoters	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

Table 3. Fold changes of methionine sulfoxide reductase (*MSR*) genes from *Arabidopsis thaliana* in the different comparisons analysed by RNA-seq. Most of these genes respond to GSH treatment and only *MSRB7* (in bold) respond to GSNO and not to GSH under our experimental conditions (Fc ≥ 2 up and down, p-value < 0.05). RNA-seq analysis was done in roots and leaves samples of 30-day-old Arabidopsis plants incubated throughout the roots for 3 h either with solutions of 1mM GSNO (NO donor) and 1mM GSH or with distilled water as controls. ↑, up-regulated genes. ↓, down-regulated genes. "-", no affected.

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**↑	NA	-	-	-	-	**74.9**↑
AtMSRB8	At4g21840	-	3.29↑	-	-	-	-	6.29 ↑
AtMSRB9	At4g21850	-	3.64 ↑	-	-	-	-	-

Figure legends

Figure 1. (A) Appearance of a 30-day-old Arabidopsis plant and the *in vitro* experimental design used to apply throughout the roots either solutions of 1mM GSNO (NO donor), 1mM GSH or distilled water (control). After 3 h of each treatment, leaves and roots were harvested and used for RNA isolation.
(B) Schematic Process flux of the experimental procedure

Figure 2. Distribution of GSNO-responsive genes from whole Arabidopsis plants in several Gene Ontology categories. Genes with putative functions were assigned to (**A)** Molecular function, (**B)** Biological process or (**C)** Cellular component categories using Gene Ontology Annotations from the TAIR database.

Figure 3. Volcano plots of significant genes in leaf and root of Arabidopsis plants after RNA-seq analysis. X-axis represents the natural logarithm of Fold Change (Fc) and Y-axis represents Log_{10} of P-value in each gene. Several breakpoints of Fc values are indicated in X-axis, where 0 would indicate "no-change". Up-regulated genes are observed in red (Fc=2.7 up) and orange (Fc=2 up), while down-regulated genes are shown in blue (Fc=2.7 down) and light blue (Fc=2 down). In black and grey colour appear genes with a slight expression-change.

Figure 4. Functional classification of GSNO-responsive genes in roots of Arabidopsis plants. Genes were classified by functional categories under the follow Gene Ontology (GO) terms: biological process (panels **A, D**), molecular function (panels **B, E**) and cellular component (panels **C, F**) and by up-regulated (panels **A-C**) or down-regulated (panels **D-F**) genes. The number of genes assigned to each functional category is expressed in percentage (%).

Figure 5. Functional classification of GSNO-responsive genes in leaves of Arabidopsis plants. Genes were classified by functional categories under the follow Gene Ontology (GO) terms: biological process (panels **A and D**), molecular function (panels **B, E**) and cellular component (panels **C, F**) and by up-regulated (panels **A-C**) or down-regulated (panels **D-F**) genes. The number of genes assigned to each functional category is expressed in percentage (%).

Figure 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 for differential expression changes analysis by qRT-PCR (red bars). Comparison of fold change of RNA-seq and qRT-PCR shows a correlation coefficient of 0.91, indicating that RNA-seq results obtained are reliable. Error bars represent the standard error of the mean. *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 dehydrogenise-like 6. *LRR* (AT2G15880): leucine-rich repeat family protein. *PEROXIDASE 31* (AT3G28200): putative peroxidise. *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 ferredoxine NADP reductase. *ELF* 4 (AT2G40080): early flowering 4. *ATBF* (AT1G49720): abscisic acid-intensive 5-like protein. *WOUNDING* (AT4G10270): wounding-responsive family protein. *HSP 70* (AT4G16660): heat shock protein 70, putative.

SUPPLEMENTARY DATA

Supplementary Fig. S1. Functional classification of GSNO-responsive genes in roots respect to leaves of Arabidopsis plants.

Supplementary Fig. S2. Functional classification of genes that respond to GSNO only in roots or leaves of Arabidopsis plants.

Supplementary Table S1. GSNO-responsive genes in Arabidopsis roots.

Supplementary Table S2. GSNO-responsive genes in Arabidopsis leaves.

Supplementary Table S3. List of organ-specific or differentially expressed genes between Arabidopsis leaves and roots.

Supplementary Table S4. Oligonucleotides used for qRT-PCR analysis.

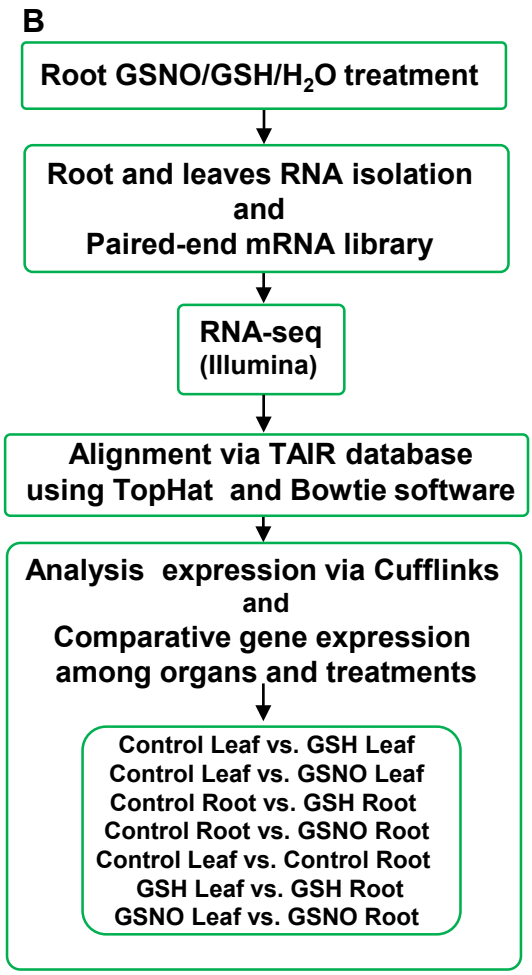


Figure 1. **(A)** Appearance of a 30-day-old Arabidopsis plant and the *in vitro* experimental design used to apply throughout the roots either solutions of 1mM GSNO (NO donor), 1mM GSH or distilled water (control). After 3 h of each treatment, leaves and roots were harvested and used for RNA isolation. **(B)** Schematic Process flux of the experimental procedure.

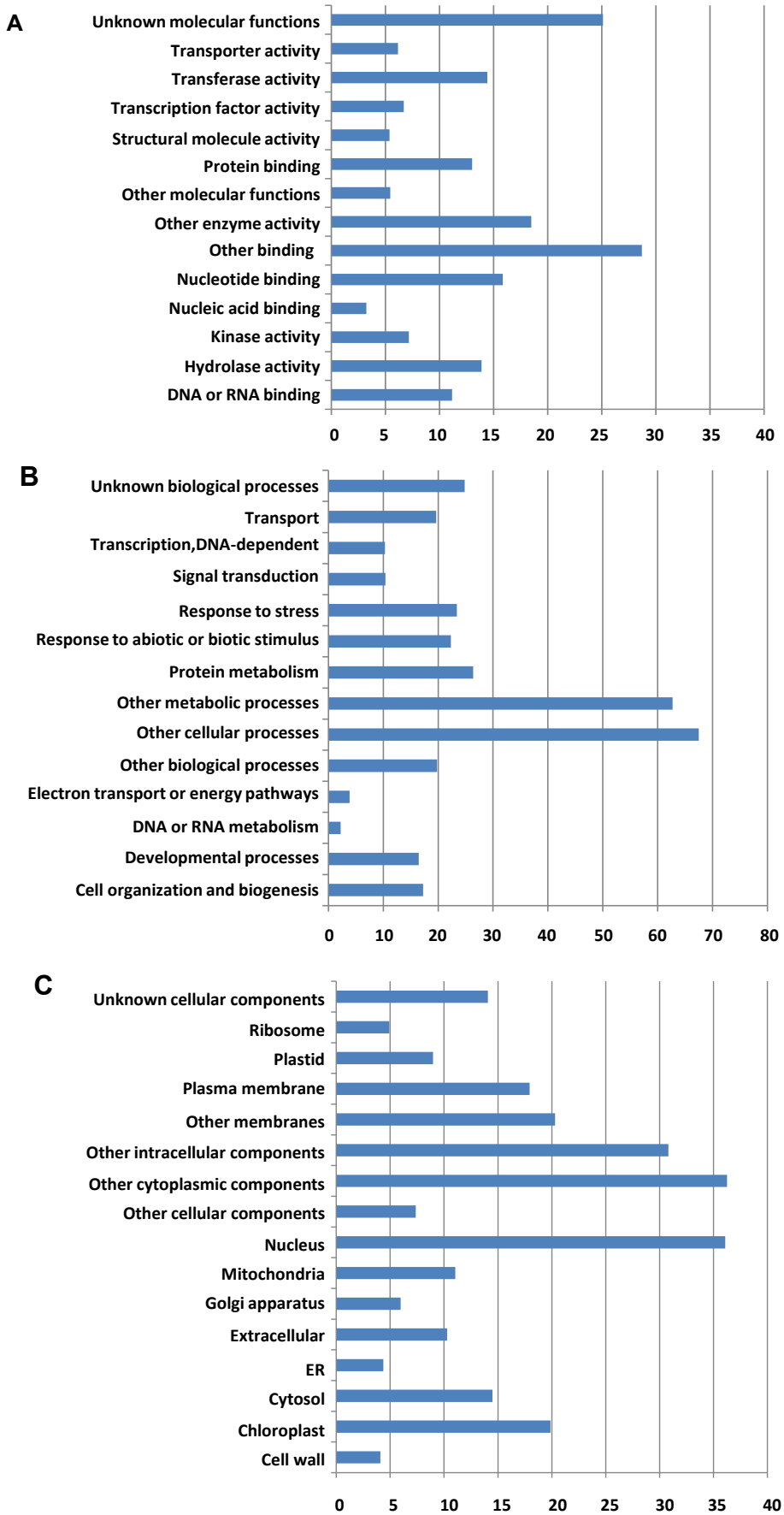

A
Unknown molecular functions
Transporter activity
Transferase activity
Transcription factor activity
Structural molecule activity
Protein binding
Other molecular functions
Other enzyme activity
Other binding
Nucleotide binding
Nucleic acid binding
Kinase activity
Hydrolase activity
DNA or RNA binding
0 5 10 15 20 25 30 35 40
B
Unknown biological processes
Transport
Transcription,DNA-dependent
Signal transduction
Response to stress
Response to abiotic or biotic stimulus
Protein metabolism
Other metabolic processes
Other cellular processes
Other biological processes
Electron transport or energy pathways
DNA or RNA metabolism
Developmental processes
Cell organization and biogenesis
0 10 20 30 40 50 60 70 80
C
Unknown cellular components
Ribosome
Plastid
Plasma membrane
Other membranes
Other intracellular components
Other cytoplasmic components
Other cellular components
Nucleus
Mitochondria
Golgi apparatus
Extracellular
ER
Cytosol
Chloroplast
Cell wall
0 5 10 15 20 25 30 35 40

Figure 2

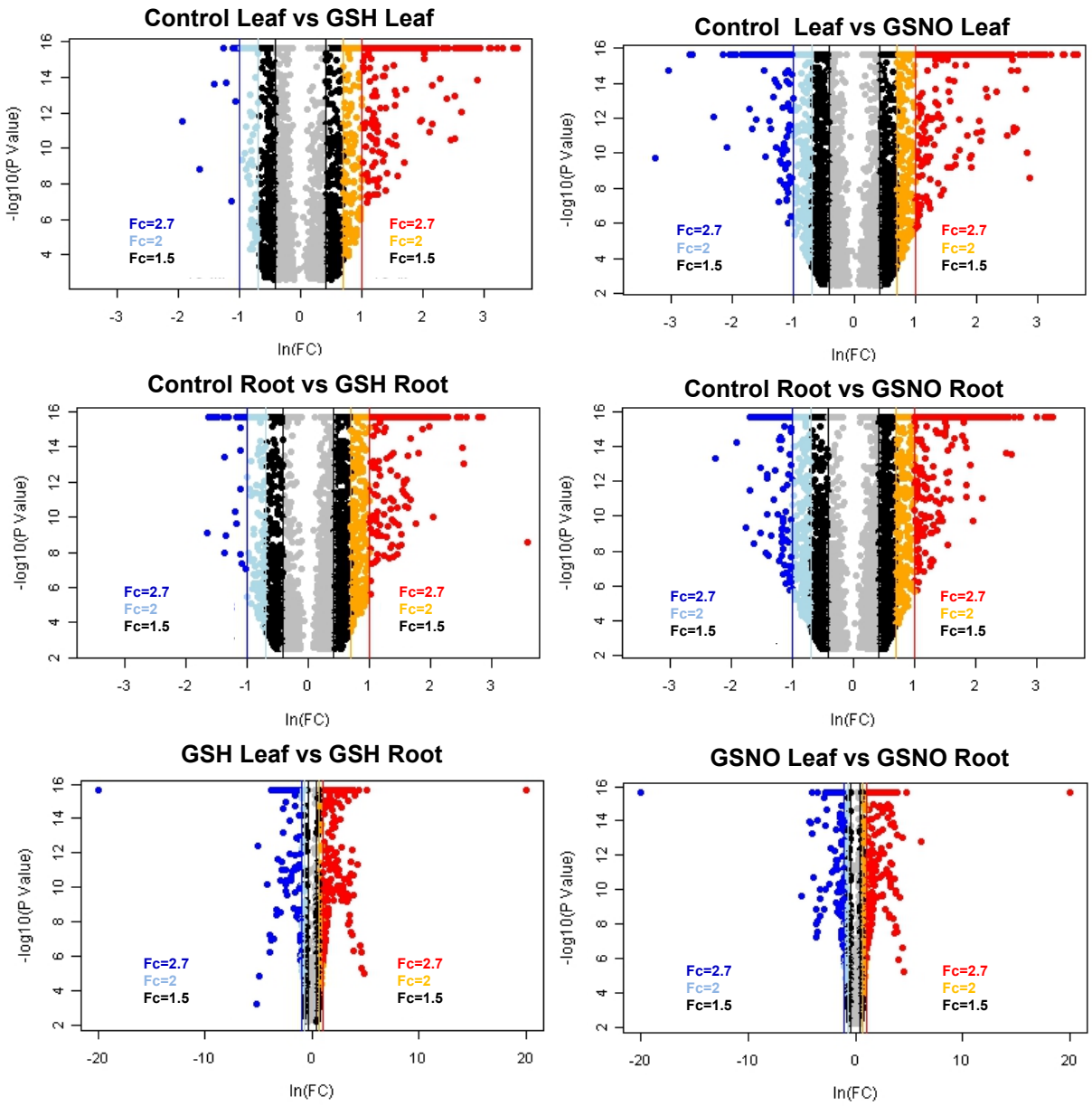
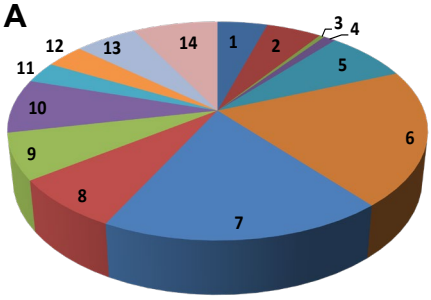

Control Leaf vs GSH Leaf
Control Leaf vs GSNO Leaf
Control Root vs GSH Root
Control Root vs GSNO Root
GSH Leaf vs GSH Root
GSNO Leaf vs GSNO Root
-log10(P Value)
ln(FC)
Fc=2.7
Fc=2
Fc=1.5

Figure 3

Root Control vs GSNO

UP-REGULATED

A



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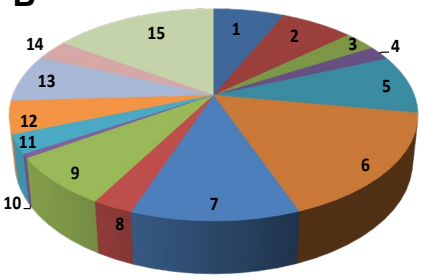

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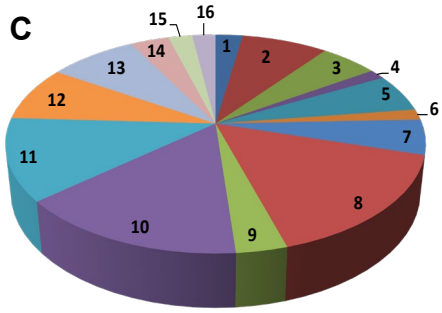
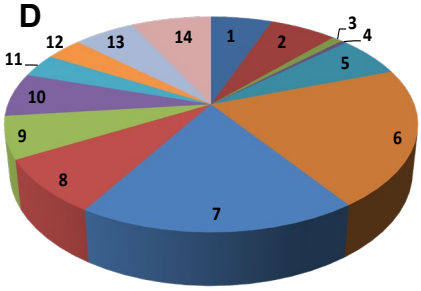


DOWN-REGULATED

D



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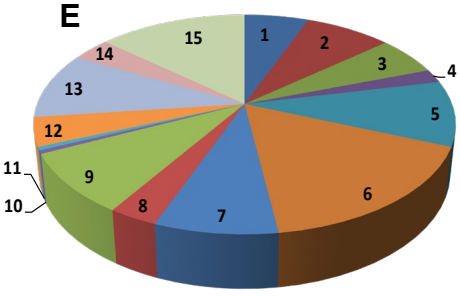
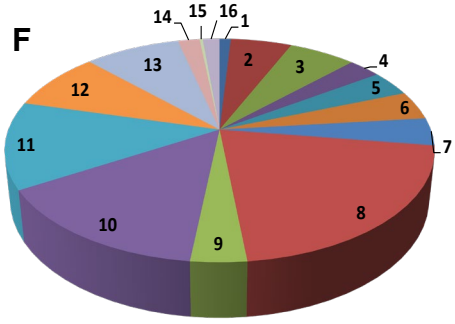


F



BIOLOGICAL PROCESS

	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

MOLECULAR FUNCTION

	GO Term	B	E
1	DNA or RNA binding	6.7	5.9
2	hydrolase activity	6.9	7.9
3	kinase 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

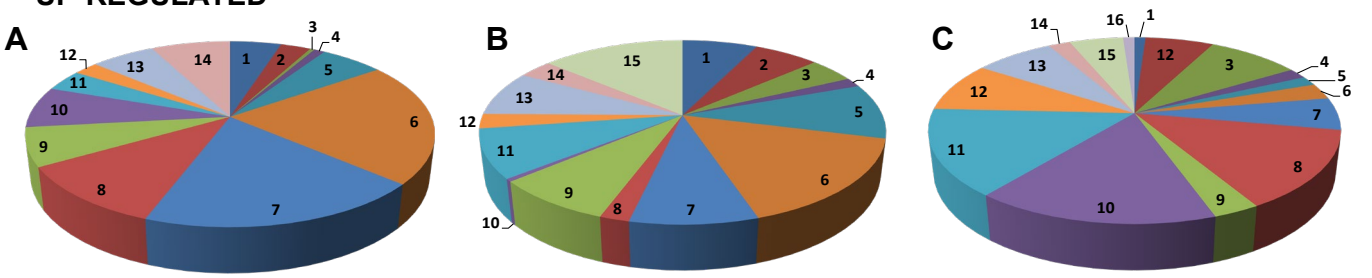
CELLULAR COMPARTMENT

	GO Term	C	F
1	cell wall	2.5	1.0
2	chloroplast	7.9	5.5
3	cytosol	5.3	6.0
4	ER	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

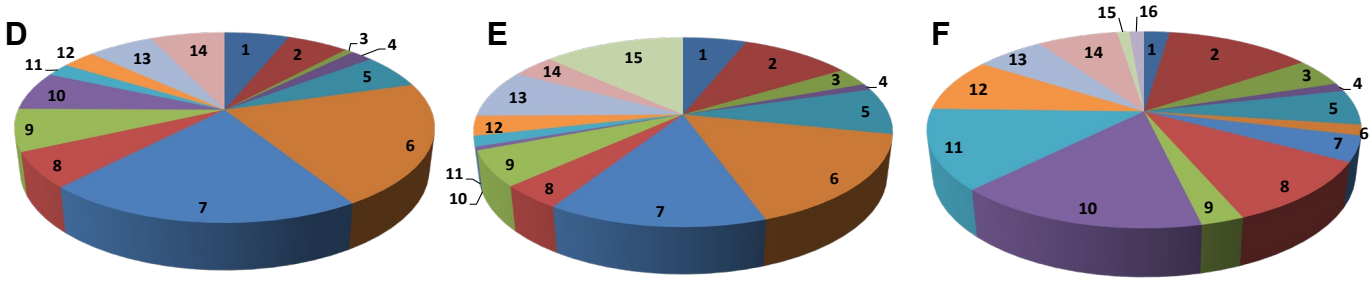
Figure 4

Leaf Control vs GSNO

UP-REGULATED



DOWN-REGULATED



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	ER	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

Figure 5

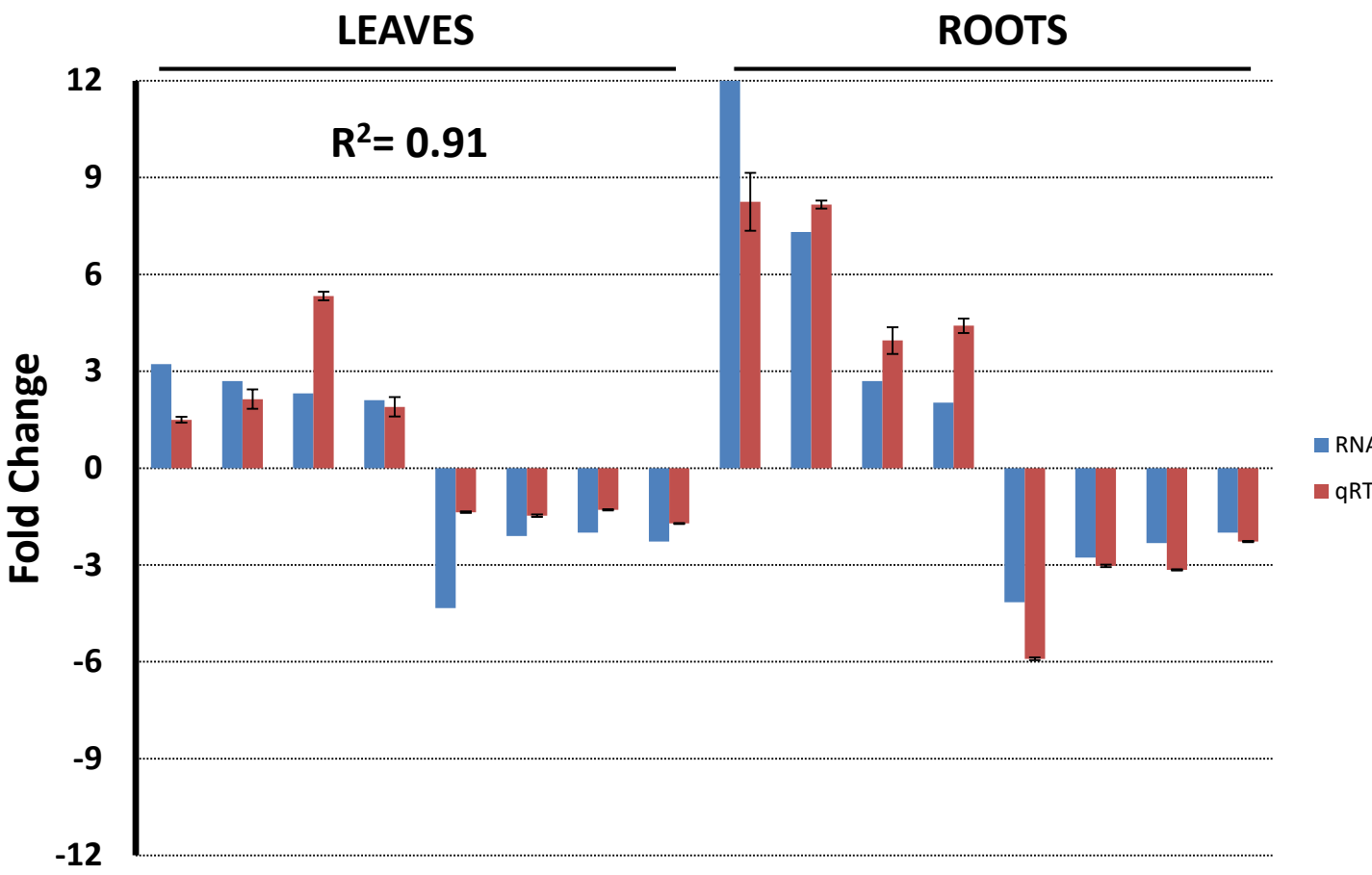


Figure 6.