Transcriptome wide identification and characterization of NO-responsive WRKY transcription factors in Arabidopsis thaliana L.

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ABSTRACT

WRKY transcription factors are important plant-specific regulatory genes characterized by one or two conserved WRKY domain(s) usually followed by a zinc-finger motif. In this study using Arabidopsis thaliana, the RNA-Seq based transcriptomic analysis showed differential expression of 33 genes encoding WRKY TFs in response to the nitric oxide (NO) donor S-Nitrosocysteine (CySNO). Interestingly, 93.9% of these TFs were up-regulated with at least 2-fold change, suggesting their putative involvement in NO mediated gene regulation. GO- analysis of all the 33 transcriptomic elements showed their putative involvement in biological processes such as abiotic stress tolerance and defense against fungal pathogens (89.39 fold enrichment). Analysis of the NO-responsive AtWRKY TFs promoter region revealed the presence of the cis-acting elements such as ABRE, EIRE, ERE, and MBS involved in osmotic stress response, maximal elicitor-mediated activation, and drought-stress regulation. The analysis of NO-responsive AtWRKY TF motifs and their comparison with rice, soybean, and tomato orthologs suggested that members of the WRKY family belonging to the same group shared similar motifs and phylogenetic tree suggested that these TFs were highly conserved. Validation of transcriptomic data through quantitative real-time-PCR showed a high correlation coefficient (0.85) indicating the high reliability and similarity of both types of analysis. Comparison of the NO-responsive and non-responsive WRKYs showed the presence of tyrosine (T) and cysteine (C) residues at a distance of 7 residues from the WRKYGQK motif which may serve as potential NO targeting sites. The RNA-Seq analysis using atwrky62 loss of function mutant and the results indicated a negative role of AtWRKY62 in NO metabolism. Furthermore, atwrky62 showed significantly less NO contents compared to wild type plants indicating putative role of AtWRKY62 in NO metabolism.

1. Introduction

Plants continuously face adverse environmental conditions that restrict their growth and productivity. In turn, plants have evolved complex coping strategies in response to adverse environmental conditions. Several reports have suggested that these regulatory mechanisms act via transcriptional activation or repression of regulatory genes (Chen et al., 2002; Kalde et al., 2003). Transcription factors (TFs) comprise a group of genes that regulate (activate or deactivate) the transcription of other genes. One of the most important and largest plant-specific TF families is the WRKY TF family. Members of this group are involved in the regulation of a number of biological processes such as plant development (Yu et al., 2012), responses to pathogen ingress (Cheong et al., 2002), nutrient deficiency, seed development and senescence (Bakshi and Oelmüller, 2014), and responses to drought and excessive salt accumulation (Seki et al., 2002). The plant-specific WRKY
TF group contains several members in different plant species including 72 in Arabidopsis (Eulgem et al., 2000), more than 100 in rice, popular and soybean (Schmutz et al., 2010; Yu et al., 2010), 68 in sorghum, 38 in a spreading earth moss (Pandey and Somssich, 2009), 35 in spike moss (Rushton et al., 2010), 80 in pines (Liu and Ekramoddoullah, 2009), and 45 in barley (Mangelsen et al., 2008). Each member of the family contains either one or two highly conserved WRKY domain(s), consisting of the heptapeptide sequence WRKYQGKQ followed by a novel zinc-finger motif, Cys(2)-His(2) (C2H2) or Cys(2)-HisCys (C2HC), located at the N-terminus (Rushton et al., 2010). Zinc finger motif; initially studied in Xenopus laevis and called as DNA/RNA binding transcription factor TFIHIA (Ginsberg et al., 1984) is a 9-fold repeated pattern of amino acids having conserved cysteine, histidine and hydrophobic residues (Miller et al., 2001). The arrangement of these residues in TFIHIA is S-X-C2-X4,5-C-X7-X8-S-X9-H-X14-H where X may be any amino acid, $ represent a hydrophobic residue while C and H represent cysteine and histidine respectively. Furthermore, based on biochemical and biological analysis of this pattern, Miller et al. (2001) named it “zinc finger” and suggested that this thirty-amino-acid motif forms a DNA binding mini-domain folded around a central tetrahedral arrangement of cysteine and histidine metal ligands (Klug and Schwabe, 1995). Based on the arrangement of zinc-finger motif and on the number of WRKY domain(s), WRKY TFs are classified into three major groups and further sub-groups (Eulgem et al., 2000; Rushton et al., 2010). Group I members typically have two WRKY domains and a C2H2 zinc-finger motif whereas Group II members have a single WRKY domain and a C2H2 zinc-finger motif. According to the pattern of their zinc-finger motif, Group II members can be further divided into Iia (C-X5-CX23-HXH), Iib (C-X5-CX23-HXH), Iic (C-X4-CX23-HXH), IId (C-X5-CX23-HXH), and Ile (C-X5-CX23-HXH). A single WRKY domain and a C2HC zinc-finger motif characterize Group III WRKY TFs. Group III WRKY TFs can also be subdivided into two types on the basis of pattern of zinc-finger motif. The structure of zinc-finger motif for subgroup IIIa is C-X7-C-X23-HXH, while that of subgroup Iib is C-X7-C-Xn-HXH (n ≥ 24). The C-terminal WRKY domain can directly bind DNA while the N-terminal cannot; instead, it assists the C-terminal in DNA binding, increasing its capacity. The heptapeptide sequence (WRKYQGK) specifically binds to consensus cis-acting elements (i.e., W-box, sequence = TTGACT/C) in the promoter region of target genes, regulating their transcription (Rushton et al., 2010).

The WRKY TF family is well studied in A. thaliana. Jiang and Deyholos (2009) found that AtWRKY25 and AtWRKY33 improve salt tolerance in this species, and Kalde et al. (2003) stated that most of the 13 AtWRKY TFs they studied responded to salicylic acid (SA) and pathogen infection. Similarly, Cheong et al. (2002) revealed that wound induction the expression of AtWRKY33 and AtWRKY40. Similarly, reports suggested that AtWRKY6 regulates senescence and plant defense (Robatzek and Somssich, 2001). The role of WRKY TFs in seed development has also been studied in A. thaliana and it was revealed that Transparent Testa Glabra2 (TTG2) that codes a WRKY TF is needed for tannin and mucilage production in the seed coat and is the first member of this family to be involved in regulation of morphogenesis (Johnson et al., 2002). Furthermore, the WRKY TF ABO3 is involved in the regulation of ABA-mediated drought tolerance in A. thaliana (Ren et al., 2010).

Biotic and/or abiotic stresses including cold, drought, salinity, heavy metal, and oxidative stress lead to the production of two types of highly reactive redox molecules; reactive oxygen species (ROS) and reactive nitrogen intermediates (RNs) (Burnistion and Wilson, 2008; Garcia-Mata and Lamattina, 2002; Kopyra and Gwóźdź, 2003). The production, signalling, and role of ROS in plants have been extensively studied (Apel and Hirt, 2004; Gill and Tuteja, 2010). However, production of NO in plants and the exact mechanism of action are still unknown. Plants produce basal levels of NO under normal conditions that significantly increase in response to biotic and/or abiotic stresses. Being short-lived and highly reactive, NO is very toxic for plants if produced in excess. Therefore, plants have a fine-tuned mechanism to regulate cellular NO level through S-nitrosylation, a post-translational modification in which an NO moiety is covalently attached to solvent-exposed cysteine thiolis to form S-nitrosothiols (SNO). This allows for the attachment of excess NO to glutathione (GSH) to form S-nitrosothioglutathione (GSNO), a non-toxic mobile reservoir of NO (Jaffrey et al., 2001). Although, the function of NO was first discovered and studied in mammals, however, in plants it has been known to regulate various developmental processes, such as germination (Beligni and Lamattina, 2000), flowering time (He et al., 2004), flower development and apical dominance (Kwon et al., 2012; Lee et al., 2008), and auxin-mediated root growth and development (Yu et al., 2014). Furthermore, NO plays a key role in plant defense during pathogen infection (Delledonne et al., 1998; Feechan et al., 2005; Yun et al., 2011).

NO-mediated transcriptional changes have been investigated in several studies that report substantial number of NO-modulated genes; several of which are regulatory proteins or TFs (Palmieri et al., 2008). Several studies involving transcriptome analysis of plants in response to different NO donors report differential expression of a plethora of genes. For example transcriptome analysis of A. thaliana leaves and roots after 3 h of 1 mM GSNO exogenous application yielded 3263 differentially expressed genes (DEGs) (Begara-Morales et al., 2014). These also include 35 TFs in which 25 TFs showed differential expression in roots while 10 in leaves. Similarly, using the whole-genome microarray analysis, Parani et al. (2004) showed differential expression of 422 genes in response to 0.1 mM and 1 mM sodium nitroprusside (SNP) in A. thaliana. In a previous study, using high throughput RNA sequencing we recorded significant variation in the expression of 6436 Arabidopsis genes in response to 1 mM S-nitroso-cysteine (CySNO within 6 h) of infiltration (Hussain et al., 2016).

WRKY TFs are unique to plants regulating various physiological functions therefore; any changes at transcriptional level in response to environmental stimuli would alter diverse functions in plants. Identification of these regulatory network components in response to CySNO (NO-donor) would aid exploration of mechanistic control of diverse processes in plants. Therefore, in the current study, we mainly focused on the transcriptome-wide analyses of NO-responsive WRKY TFs in A. thaliana. The functional classification of these TFs indicated their putative involvement in different biological and molecular processes. Overall, this study further elucidated the importance and putative involvement of WRKY TFs in plant’s stress tolerance and defense.

2. Materials and methods

2.1. Plant material

Seeds of A. thaliana accession Col-0 (wild type) were grown in trays under 16:8 h light: dark conditions at 23 ± 2 °C for four weeks and then infiltrated with 1 mM CySNO at the abaxial side of leaves to induce robust changes in gene expression. Application of 1 mM CySNO is a standard dose used in different studies such as (Martínez-Ruiz and Lamas, 2004; Polverari et al., 2003). Leaf samples were collected and processed as described by Hussain et al. (2016). Briefly, three leaf samples were collected from each replicate (three replicates in total) and pooled together for RNA-seq analysis.

2.2. Plant growth and exposure to nitrosative stress

Seeds of A. thaliana accession Col-0 (wild type) and atwrky62 (GK-016H10) loss of function mutant were obtained from Arabidopsis Biological Resource Center ([ABRC] https://abrc.osu.edu/). AtWRKY62 was selected for in vivo analysis based on having highest fold change in response to CySNO. For nitrosative stress assay, wild type and atwrky62 mutant seeds were germinated in square plates (12 cm²) containing either half-strength MS medium for control treatment or half-strength MS medium supplemented with 1 mM CySNO or GSNO for nitrosative stress conditions. A. thaliana accession Col-0 (wild type) and atwrky62 (GK-016H10) loss of function mutant were obtained from Arabidopsis Biological Resource Center ([ABRC] https://abrc.osu.edu/). AtWRKY62 was selected for in vivo analysis based on having highest fold change in response to CySNO. For nitrosative stress assay, wild type and atwrky62 mutant seeds were germinated in square plates (12 cm²) containing either half-strength MS medium for control treatment or half-strength MS medium supplemented with 1 mM CySNO or GSNO for nitrosative stress conditions.
stress application. Plants were grown under conditions mentioned in Section 2.1 for two weeks. Cotyledon development frequency (CDF), shoot and root length were recorded 2 weeks post-treatment. The term CDF was used to mention the number of developed green seedlings (Yun et al., 2011).

2.3. Transcriptome-wide identification of WRKY genes

- Extraction of RNA for RNA-Seq and other analysis to identify DEGs has already been described in Hussain et al. (2016). Briefly, RNA from fresh leaves was extracted using RNeasy Plant Mini Kit (Qiagen USA) according to manufacturer’s instructions. RNA integrity and purity was analyzed (Agilent 2100 Bio-analyzer, Agilent) and RNA libraries were generated using TruSeq™ RNA library prep kit (Illumina USA). Single stranded cDNA was synthesized using hexamer priming of mRNA to generate double stranded cDNA libraries, which were then quantified using KAPA library quantification kit (Illumina USA), and sequenced through HiSeq-2500 sequencer (Illumina). The raw sequence reads were further processed to identify high quality reads using threshold level Q20 > 40% and reads with > 10% ambiguous bases or with Q20 < 40% were removed (Patel and Jain, 2012). The TopHat (Trapnell et al., 2009) was used to align the high-quality reads against A. thaliana reference genome with default values and gene annotation obtained from Ensembl (release 15) (Flicek et al., 2014). Abundance of the transcripts was inferred using Cufflinks package v2.2.1 (Trapnell et al., 2010). The gene and transcript expression levels were calculated under control and treated conditions and were tested for significant difference using Cuffdiff v.2.2.1 (Trapnell et al., 2010) to identify DEGs. The genes showing significant differential expression (Q < 0.05) were selected for further analysis.

- The DEGs were mapped against the Arabidopsis “Ath_AGILOCUS.TAIR10_Aug2012.m02” database using MapMan 3.6.0RC (Thimm et al., 2004; Udadi et al., 2005). MapMan uses the hierarchical “BIN”-based ontology classification system in which unique BIN numbers are allocated to different pathways and processes. Based on BIN number (27.3.32), representing “WRKY domain transcription factor family”, 33 NO-responsive WRKY TFs (31 up- and 2 down-regulated) were identified.

- A heatmap visualizing the expression differences between CySNO treated and control samples with dendrogram representing the hierarchical clustering was generated using FPKM values with R version 3.3.1.

2.4. Correlation, chromosomal location, and distribution of NO-responsive WRKY genes

- The presence of the WRKY domain (IPR003657) was verified in the NO-responsive TF genes using the protein sequence analysis and classification program InterPro (http://www.ebi.ac.uk/interpro/) (Jones et al., 2014). The location of NO-responsive AtWRKY genes on chromosomes was analyzed by querying the NO-responsive AtWRKY sequences against A. thaliana whole-genome data using Phytozome 11.0 (https://phytozome.jgi.doe.gov/pz/portal.html). Correlations among NO-responsive AtWRKY TFs were also determined to evaluate any possible interaction among these genes under nitrosative stress.

2.5. Gene ontology (GO) analysis

- To further understand the role of NO-responsive WRKY TFs in biological processes, an analysis of associated GO terms was done using the Panther database web interface (http://pantherdb.org) “PANTHER version 11.1 Released 2016–10–24”. The IDs of the 33 differentially expressed WRKY TFs were searched through search field while A. thaliana was selected as organism and analysis method was selected as “Statistical overrepresentation test” using default settings. Bonferroni correction was selected to correct p-Values for multiple hypothesis testing. Thus the analyzed data sets for “GO biological process” and “GO molecular function” were studied and GO terms with an enrichment p-Value < 0.05 were downloaded for each annotation data set, respectively (Mi et al., 2013). GO terms with p < 0.05 were selected, compiled, and displayed using pie charts.

2.6. Classification and phylogenetic analysis of NO-responsive AtWRKY genes

- Based on the number of WRKY domains and on the pattern of zinc-finger motifs, WRKY TF genes were classified into Group I, II, or III as described in the introduction section. To investigate role of group-specific WRKYs, the protein sequences of NO-responsive AtWRKY TFs were aligned using MEGA 7 (Kumar et al., 2016). The evolutionary conservation of NO-responsive AtWRKY TFs throughout the plant kingdom and the presence of gene orthologs was evaluated using 12 NO-responsive AtWRKY TFs (a subset of WRKYs were selected to analyze and compare easily) representing each group – Group I: AtWRKY3, AtWRKY4, AtWRKY25, AtWRKY26, and AtWRKY33; Group II: AtWRKY15, AtWRKY36, AtWRKY40, AtWRKY61, and AtWRKY65; and Group III: AtWRKY55 and At4g23810. The sequences of these proteins were queried against rice (Oryza sativa), soybean (Glycine max), and tomato (Solanum lycopersicum L.) proteins, using the basic local alignment search tool for proteins (blastp) suite with default values in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). The retrieved protein sequences (best-hits) were aligned using ClustalW tool and the resulting alignment was then used to create a neighbor-joining phylogenetic tree in MEGA7, whose branching pattern was evaluated by running 1000 bootstrap replicates.

- To evaluate the structural divergence of WRKY TFs from different species, the conservation of protein motifs was evaluated using the Multiple Expectation Maximization for Motif Elicitation program (Bailey and Elkan, 1994). The parameters used were those described in (Fan et al., 2015).

2.7. Promoter analysis of NO-responsive AtWRKY TF genes

- TFs have the ability to bind DNA at specific conserved sequences in the promoter region called regulatory or consensus cis-acting elements (Palmieri et al., 2008). These sequences play a key role in the regulation of gene function. Therefore, to predict the putative regulatory role of NO-responsive AtWRKY TF genes, we retrieved the genomic sequences 1.5 kb upstream of the transcription initiation site of all the NO-responsive AtWRKY TF genes from The Arabidopsis Information Resource (TAIR) center (https://www.arabidopsis.org/). All sequences were surveyed for regulatory elements in their promoter regions using Plant cis-acting Regulatory Element (PlantCARE) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002). The cis-acting elements obtained were manually screened and only those that were involved in abiotic and biotic stresses were mapped (on both strands) using the Regulatory Sequence Analysis Tool (Medina-Rivera et al., 2015).

2.8. Validation of RNA-Seq results through quantitative real-time-PCR

- To validate CySNO-mediated transcriptional changes in the AtWRKY TFs obtained from the RNA-Seq mediated transcriptomic data, we selected 16 NO-responsive AtWRKY TFs for quantitative real-time-PCR (qRT-PCR) (Supplementary Table S4). Total RNA was extracted using Trizol™ reagent (Ambion Life Technologies, USA) in triplicates. Complementary DNA was synthesized from 2 μg of total RNA with good integrity and purity using DiaStar™ RT Kit (SolGent Korea) following manufacturer’s instructions. A two-step qRT-PCR reaction was
performed in an Illumina Eco™ system (Illumina, USA) to quantify transcript accumulation. The reaction mixture contained 10 µl 2 x Quantspeed SYBR Kit (PhilE Korea Korea) and 1 µl each primer (10 µM). The thermal cycling conditions comprised an initial denaturation at 95 °C for 2 min and 40 cycles at 95 °C for 10 s and 60 °C for 30 s respectively. Melting curves were then analyzed at 60–95 °C to verify amplicon specificity for each primer pair. Actin was used as the internal reference gene.

The fold change obtained was compared with RNA-seq analysis by correlation and calculating cronbach’s alpha using student statistic 9 software.

2.9. SNO measurement

To determine the role of AtWRKY62 in NO metabolism, we quantified cellular S-nitrosothiol (SNO) levels in wild type and atwrky62 a loss-of-function mutant as described by (Imran et al., 2016). Briefly, plant samples from wild type and atwrky62 were ground in KPI buffer (pH 5.5). Total protein content in the samples was quantified using Bradford assay. SNO levels were determined for all the plants samples and various CySNO standards (ranging from 50 to 1 µM) in at least three replicates. SNO levels were measured using NO analyzer (NOA-280i, Sievers, USA) by injecting 100 µl of sample into the purge vessel. A standard curve was constructed to measure protein concentration in all the samples via Bradford assay. To quantify SNO content (pmol µg⁻¹ protein) values obtained for samples were compared to those obtained for the various CySNO standards.

2.10. Comparison of NO-responsive and non-responsive WRKY TFs

To examine any difference in residual or structural changes between NO-responsive and non-responsive WRKY TFs, four NO-responsive WRKYs having highest fold change were selected and their protein sequences were aligned with four NO non-responsive WRKYs using ClustalW in MEGA 7 (Kumar et al., 2016). To study if the cysteine or tyrosine residues were solvent exposed, we analyzed the protein sequences to predict the 3D structure of both NO-responsive and non-responsive WRKYs using RaptorX with default parameters (Kallberg et al., 2012).

The location of NO non-responsive AtWRKY genes on chromosomes was analyzed by using Phytozome 11.0 (https://phytozome.jgi.doe.gov/pz/portal.html).

3. Results

3.1. AtWRKY62 negatively regulates shoot and root growth

To examine possible role of NO-induced WRKYs in plant growth and development, the mutant line atwrky62 was screened for its phenotype under control and nitrosative stress conditions. Furthermore, as discussed in section 2.2, the expression of AtWRKY62 increased by 167 folds (highest among all NO-responsive WRKYs) following nitrosative stress treatment, we were further interested to see the phenotype of atwrky62 under nitrosative stress induced by 1 mM CySNO and GSNO. Interestingly, the mutation showed a positive impact on various developmental features as the mutant seeds germinated much faster and with a greater germination frequency compared to WT plants (Fig. 1A). The vigorous growth characters of the mutant plant could even be seen at later stages of development as these plants produced significantly longer roots and shoots compared to WT plants (Fig. 1B&C). Several studies have shown a significant correlation between various developmental processes and cellular nitrosative conditions (Corpas et al., 2006; Sanz et al., 2015). Therefore, to further elucidate the indicative relationship between ATWRKY62 and nitrosative stress we quantified the total SNO content of the mutant plants. Interestingly, the atwrky62 plants accumulated significantly lower content of S-nitrosothiols compared to the WT plants (Fig. 1E).

3.2. Transcriptome-wide identification, correlation and chromosomal location of NO-responsive AtWRKY TFs

In a previous study using RNA-Seq mediated transcriptome analysis we identified 6436 DEGs (including 3448 up- and 2988 down-regulated genes) in A. thaliana leaves infiltrated with 1 mM CySNO (Hussain et al., 2016). To further study the induced or reduced expression of WRKY TFs during nitrosative stress in plants, 33 AtWRKY TFs (31 up- and 2 down-regulated) showing differential responses to CySNO were identified in the present study. The Heatmap displays FPKM values for the NO-responsive WRKYs (Cuffdiff p-value < 0.05) and shows 31 up-regulated and 2 down-regulated WRKY TFs following CySNO treatment (Fig. 2A). Detailed information on fold change and gene annotation is presented in Supplementary Table S1.

A gene’s location on the chromosome is important to understand the variation and evolution of a particular trait in an organism (Rockman et al., 2010). NO-responsive AtWRKY TFs were distributed across the five A. thaliana chromosomes, with chromosome 4 presenting the highest number of these TFs and chromosome 3 the lowest (Fig. 2B; Table S2). For convenience, the names of the TFs described in TAIR were used for all the AtWRKY TFs presented here. The peptide length of TFs ranged from 241 amino acids (AtWRKY63) to 514 (AtWRKY4). The details of NO-responsive AtWRKY TFs, including their names, length, chromosome number, and start and end location on the chromosome, is given in Supplementary Table S2.

The correlation analysis performed among NO-responsive WRKY TFs expression is displayed in Fig. 2C (the darker the red color the higher the correlation between TFs expression). After manually checking all AtWRKY TFs presenting the highest correlation values (i.e., 1) for significance, AtWRKY48 showed the highest number of significant positive correlations with 13 other NO-responsive AtWRKYs majority of which (69.2%) were from Group II. While lowest number of positive correlations was found for AtWRKY30, AtWRKY75, AtWRKY53, AtWRKY28 and AtWRKY4, which had only significant correlation with AtWRKY3 (1 out of the two down-regulated WRKY TF). Similarly, highest number (14) of significant negative correlations was found for AtWRKY8, which is a member of Group Ic WRKY TFs.

3.3. GO terms for NO-responsive AtWRKY TFs

The putative functions of NO-induced WRKY TFs were analyzed through GO, consortium database (http://geneontology.org/) using A. thaliana genome as reference. The accessions of the 33 NO-responsive AtWRKY TFs were successfully found in the reference annotation of the Panther DB. Within molecular functions terms, GO fold enrichment (FE) for sequence-specific DNA binding (53.4 FE) and regulatory regions DNA binding (23.84 FE) were the major GO categories assigned (Fig. 3A). Among the GO terms for biological functions, GO enrichment for chitin response (89.39 FE) was the major category, which means they are associated to response to fungal stimuli (Fig. 3B; Supplementary Table S3). The second major category based on fold enrichment was response to organo-nitrogen compounds (73.13 FE), which means AtWRKY TFs may also play a key role in nitrogen metabolism. Terms related to heat acclimation (67.04 FE), external stimuli (63.1 FE), SA (29.61 FE), and response to bacteria (28.73 FE) were also assigned (Fig. 3B). A detailed list of GO terms for biological processes is given in Supplementary Table S3. Overall, GO-enrichment analysis results suggested a putative regulatory role for NO-responsive AtWRKY TFs in plant defense and environmental stress resistance.

3.4. Classification, motif composition, and phylogenetic analysis

Protein sequences of all the NO-responsive AtWRKY TFs were aligned to classify them into the three main groups: five into Group I, 21
Group I AtWRKY TFs had two WRKY domains followed by a HXH type of zinc-finger motif, Group II AtWRKY TFs had a single WRKY domain followed by an identical HXH zinc-finger motif, and Group III AtWRKY TFs had a single WRKY domain followed by HXC type of zinc-finger motif (Fig. 4; Table S1).

To understand whether NO-responsive WRKY TFs are evolutionarily conserved among plant species or if their response is species specific, we evaluated the evolutionary relationships among 12 randomly
selected NO-responsive AtWRKY TFs (5 from Group I, 5 from Group II, and 2 from Group III) and their orthologs in rice, soybean, and tomato. Results indicated that all Group I, Group II, or Group III WRKY TFs clustered together irrespective of species, suggesting that their function is conserved among different species (Supplementary Fig. S1). The analysis of motif composition revealed that the WRKY domain (motif 1) was followed by the zinc-finger motif (motif 2) in all WRKY TFs (Supplementary Fig. S1), which was expected as these two motifs characterize WRKY family members. The second WRKY domain (motif 3) and the zinc-finger motifs 4, 5, and 8 were only found in Group I WRKY TFs. Motif 10 was found in 50% of Group II WRKY TFs while in Group I it was only found in AtWRKY3 and AtWRKY4, as well as in their orthologs in soybean (Supplementary Fig. S1). Motifs' detailed compositions are given in Supplementary Fig. S2. Overall, a similar WRKY TFs composition was found in A. thaliana, rice, soybean, and tomato indicating that these motifs are conserved and that these genes may

Fig. 2. Differentially expressed Arabidopsis thaliana WRKY transcription factors (TFs) in response to 1 mM S-nitrosocysteine. (A) Heatmap showing the expression patterns of transcriptome-wide differentially expressed AtWRKY TFs. (B) Chromosomal distribution of the differentially expressed AtWRKY TFs. (C) Correlation among NO-responsive AtWRKY TFs.
Fig. 3. Gene ontology (GO) analysis of CySNO-induced Arabidopsis thaliana WRKY TFs. (A) The IDs of the NO-induced WRKY TFs were inserted into the search field while selecting Arabidopsis thaliana as organism in the Panther classification system (http://pantherdb.org/webservices/go/overrep.jsp). (B) GO terms for biological processes. Only TFs associated with important biological processes are presented here. The values represent fold enrichment. The detail list for biological processes assigned is given in Supplementary Table S3.
have similar functions in other species.

3.5. Promoter analysis for cis-regulatory elements

cis-regulatory elements also called cis-acting elements are non-coding DNA sequences that have the capability to bind to specific sequences in promoter of genes and are crucial for gene regulation (Wittkopp and Kalay, 2012). Most importantly, these elements are reported to play a major role in the transcriptional control of defense-related genes, such as those involved in SA and jasmonic acid signaling (Caarls et al., 2015). Therefore, we searched for cis-regulatory elements within the promoter regions of NO-responsive AtWRKY TF genes. The cis-elements that were involved in both abiotic and biotic stresses were then mapped (Fig. 5), which revealed both types of W-box elements (TTGACC and TTGACT; Rushton et al., 2010) in the promoter regions of NO-responsive AtWRKY TFs genes. For convenience, they were named W-box1 (TTGACC) and W-box2 (TTGACT). W-box2 was found in 82.3% and W-box1 in 55% of the genes studied. The other common motif, CGTCA, was found in 76% of the genes, MBS elements in 50%, and ABRE in 44% (Fig. 5).

3.6. Comparing NO-responsive and NO-non responsive WRKY TFs

*Arabidopsis thaliana* expresses 72 known and putative WRKY genes. We found that the expression of only 33 WRKY genes changed following CySNO treatment. Though this number is big enough to establish that NO mediates multiple processes in Arabidopsis through transcriptional regulation of WRKY genes, it is still less than half of the total number of WRKY genes in this species. This tempted us to analyze the NO-responsive and non-responsive WRKY TFs and interestingly we found an intriguing difference. Most of the nitric oxide responsive WRKY TFs have either a tyrosine (Y) or cysteine (C) residue at the seventh position after the C-terminal WRKYGQK motif, whereas the NO-nonresponsive WRKY TFs did not have Y or C residues (Fig. 6A). Furthermore, analysis of the predicted 3D structure showed that the tyrosine and cysteine residues of these proteins are solvent exposed and may serve as potential targets for modification by nitric oxide via tyrosine nitration and cysteine S-nitrosylation respectively (Fig. 6B). As described earlier, the NO-responsive WRKY TFs were mostly located on chromosome 4. On the other hand, the non-responsive WRKY TFs were abundant on chromosomes 1 and 5 (Supplementary Fig. S2).

3.7. Validation of cySNO-induced transcriptional changes in AtWRKY TFs using qRT-PCR

To further confirm CySNO-mediated transcriptional changes in WRKY TFs, 16 AtWRKY TFs involved in different biological processes were selected for quantitative polymerase chain reaction (qPCR) validation. These include those involved in plant defense (AtWRKY4, AtWRKY17, AtWRKY40, AtWRKY48, AtWRKY61, and AtWRKY62;
Fig. 5. Analysis of Arabidopsis thaliana WRKY TFs promoter regions. Promoter sequences 1.5 kb upstream the transcription initiation sites were retrieved from the Arabidopsis Information Resource Center (https://www.arabidopsis.org/index.jsp) and analyzed through PlantCARE. The resulting regulatory elements were mapped using RSAT (http://floresta.eead.csic.es/rsat/).

Fig. 7A), hormone signaling (AtWRKY23, AtWRKY48, AtWRKY63, and AtWRKY47; Fig. 7B), development (AtWRKY22 and AtWRKY65, Fig. 7C), abiotic stress tolerance (AtWRKY33, AtWRKY55, AtWRKY69, AtWRKY3; Fig. 7D) and three with unknown function were selected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The transcript accumulation determined 6 h after 1 mM CySNO infiltration (Fig. 7), and high correlation coefficient (R = 0.857) between the RNA-Seq and qRT-PCR datasets showed that RNA-Seq analysis was
highly reliable.

4. Discussion

NO is a redox active, small, and important signaling molecule, that acts as biological messenger in both animals and plants (Delledonne et al., 1998; Wendehenne et al., 2001). In the last couple of decades, extensive research on NO biology has reported it to be involved in several physiological processes in plants, including stomatal regulation, seed germination, disease resistance, and plant responses to abiotic stresses (Delledonne et al., 1998; Durner et al., 1998; García-Mata and Lamattina, 2002; Zhao et al., 2004). NO can control physiological processes by directly controlling gene transcription and NO-mediated transcriptional changes have been analyzed in Arabidopsis thaliana using microarrays (Parani et al., 2004), RNA-Seq (Hussain et al., 2016), amplified fragment length polymorphisms (Polverari et al., 2003), and qRT-PCR (Huang et al., 2002).

The WRKY TFs is one of the largest plant-specific TF family. Members of this family are involved in regulating multiple processes including development and stress related responses. Several scientific studies involving transcriptomic analysis report differential expression of WRKY TFs in response to different stimuli such as salts, drought, pathogen inoculation, application of growth hormones, oxidative (e.g. H₂O₂ and ozone) and nitrosative stresses (e.g. CysNO, GSNO and SNP). A comparison of such different studies has been presented in Table 1. We found more than 85% of our differentially expressed WRKY TFs in data set published by Xu et al. (2015) representing ozone-induced differentially expressed genes mostly following the same expression pattern. Another data set published by Schluttenhofer et al. (2014) representing differentially expressed WRKY TFs in response to jasmonic acid contained 61% of the WRKY TFs identified in this study. Similarly, WRKY23, WRKY28, WRKY30 and WRKY55 were also found in a dataset representing GSNO-induced transcriptomic changes in Arabidopsis thaliana (Begara-Morales et al., 2014). Interestingly, we observed a close correlation between expression trends of WRKY genes from our study and Xu et al. (2015) where gene expression was induced by ozone (O₃). All the WRKY genes that were up-regulated by ozone treatment were up-regulated by CysNO treatment in our study. AtWRKY3 (AT2G03340) was down-regulated in both the studies. Similarly, in another study involving microarray analysis of Arabidopsis plants following treatment with 0.5 mM SNP found high correlation of gene expression patterns with those in O₃–induced microarray data set (ArrayExpress accession number E-MEXP-342). This indicates that NO- and O₃–induced changes in gene expression overlap with each other. In the present study, we focused on plant-specific WRKY TFs to understand NO-mediated early regulatory responses in plants. We identified 33 AtWRKYs that showed differential responses to CySNO as NO donor (Fig. 1A; Supplementary Table S1). Previous studies also reported the up-regulation of WRKY TFs in response to wounding, pathogens, or abiotic stresses such as cold and drought (Eulgem et al., 2000; Rizhsky et al., 2002). These stress conditions lead to the production of RNIs (Garcia and Hirt, 2014; Kopyra and Gwóźdź, 2003). Many NO-mediated biological functions are the direct consequence of the chemical interaction between NO and/or RNIs with the target proteins (Yun et al., 2011). A majority (26%) of the NO-responsive AtWRKYS were localized to chromosome 4 (Fig. 1B). Studies showed that WRKY TFs binding sites (W-boxes) often occur in clusters within the promoters, suggesting a putative synergistic action between WRKY...
Fig. 7. Validation of RNA-Seq results through quantitative real-time PCR (qRT-PCR) analysis. Sixteen *Arabidopsis thaliana* WRKY TFs showing differential expression in response to CySNO in the RNA-Seq transcriptomic analysis (black bars) were selected for validation through qRT-PCR (white bars) analysis. These genes were putatively involved in (A) Plant defense, (B) Hormone signaling, (C) Plant development, and (D) Abiotic stress tolerance as per TAIR (https://www.arabidopsis.org/index.jsp) annotation. The high correlation coefficient (Cronbach’s alpha = 0.857) indicated RNA-Seq analysis was highly reliable. Error bars indicate standard error (± SE, n = 3).
family members and/or other TFs (Maleck et al., 2000). Therefore, some chromosomes might have more W-box sequences than others. We also determined correlation to measure the association between NO-responsive AtWRKY TFs and found significant correlation among the different NO-responsive AtWRKYs (Fig. 1C). AtWRKY48, which showed the highest number of positive correlations (with 13 different NO-responsive AtWRKYs) and involved in plant defense response is induced by the bacterial pathogen *Pseudomonas syringae* (Xing et al., 2008). Majority of these interactions were related to Group II AtWRKYs that counted 69.2% of total associations. These include some important genes related to plant defense such as AtWRKY40 which is pathogen induced transcription factor that binds with W-box sequences in vitro and form protein complexes with AtWRKY63 (Van Aken et al., 2013). Co-expression of this TF with WRKY18 act as positive regulator of effector-triggered immunity as wrky18 and wrky40 double mutants showed enhanced susceptibility towards bacterial pathogen *Pseudomonas syringae* DC3000 expressing AvrRPS4 effector (Schon et al., 2013). Similarly, AtWRKY17 and AtWRKY11 that showed highest positive correlation with AtWRKY48 are reportedly involved in negative regulation of basal resistance in *A. thaliana* (Journot-Catalino et al., 2006). Furthermore, another interesting association is with AtWRKY33 that is reported to mediate two important pathways SA and JA interceding the cross-talk between the two key players in plant defense (Zheng et al., 2006).

The GO terms assignment for molecular function revealed 18.54 fold enrichment for NO-responsive AtWRKY TFs putatively involved in calmodulin binding (Fig. 2A). As the role of calmodulin in the regulation of protein phosphorylation is well established (Schubart et al., 1980), this finding supports the hypothesis that NO might cause substantial post-translational changes in certain proteins. Literature reports also suggest the transfer of NO bioactivity in biological system through S-nitrosylation (Yun et al., 2016). Our results also suggested the

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presence of two closely spaced Cysteine residues that were found in 80% of the NO-responsive AtWRKY TF protein sequences (Fig. 3) that can be potential targets for protein S-nitrosylation as suggested by Zaffagnini et al. (2013) who reported that position and presence of cysteine residues at short distances presents their enhance potential for protein S-nitrosylation. Similarly, Palmieri et al. (2008) reported that NO can alter TFs DNA binding affinity by phosphorylation or by protein-S-nitrosylation. For example, S-nitrosylation modulates the activity of the thiol-containing transcriptional activator OxyR, which, upon oxidation, controls the expression of genes involved in hydrogen peroxide detoxification (Hausladen et al., 1996). The GO terms found for biological functions suggested NO might be involved in several physiological processes. As the majority (35.3%) of TFs were assigned to chitin response, they might be involved in defense responses against fungi (as chitin is the characteristic component of fungal cell walls). Some of the NO-responsive WRKY TFs are already validated for their role in fungal resistance in Arabidopsis or other plant species through biological experiments. For example, A. thaliana HOOKLESS1 (HLS1) modulates the expression of AtWRKY33 against fungal and bacterial pathogens (Liao et al., 2016). Similarly, AtWRKY40 in association with AtWRKY18 positively regulates defense response to Botrytis cinerea (B. cinerea) (Schon et al., 2013). AtWRKY50 regulates SA- and low oleic acid-dependent repression of JA pathway leading to enhanced resistance to Alternaria brassicicola but enhanced susceptibility to B. cinerea (Gao et al., 2011). Ortholog of AtWRKY25 in Gossypium hirsutum (GhWRKY25) negatively regulates resistance to B. cinerea infection in transgenic tobacco (Liu et al., 2016). The second major category within biological functions to which AtWRKY TFs were assigned was response to organo-nitrogen compounds with fold enrichment of 73.13 followed by heat acclimation (67.04) (Fig. 2B), all suggesting a possible role for NO-induced AtWRKY TFs in biotic and/or abiotic stress transcriptional regulation, mediating communication between defense systems. In a previous study, involving microarray based analysis and using Genomatix Gene2Promoter and MatInspector, Palmieri et al. (2008) reported 8 families of transcription factor binding sites (TFBS) including WRKY among others in the promoter regions of NO-regulated genes. This suggests a strong cross-talk between NO and WRKY TFs in regulation of genes responsible for nitrogen metabolism during NO signal transduction. Probably, that is the reason that majority (94%) of NO-induced WRKY TFs were up-regulated in response to NO donor (CySNO) (Fig. 1A; Supplementary Table S1).

We also found AtWRKY TFs putatively associated to SA response, the key player in plant’s defense system (Fig. 2B; Supplementary Table S3). Previous studies also reported several AtWRKY TFs that were differentially regulated in response to bacterial infection or SA (Dong et al., 2003). Yu et al. (2001) suggested that WRKY TFs regulate the expression of the transcriptional co-ordinator NPR1, which has been suggested to acts as an SA receptor in plant defense as it induces the expression of defense related genes (Wu et al., 2012).

The presence of all three major Groups of WRKY TFs in NO-responsive AtWRKYs indicated that NO-response is not group-specific and is effective at broader range. The phylogenetic tree constructed for members of the three major AtWRKY TF groups and their orthologs in rice, soybean, and tomato showed that they were evolutionarily conserved. Motif composition analysis also indicated WRKY family members within the same group (Fig. 4) suggesting that these WRKY members have similar function in Arabidopsis, rice, soybean and tomato and that they shared most motifs. A similar study using salt responsive members of WRKY TFs in cotton and their orthologs in Arabidopsis, rice and soybean suggested similar motif composition within the same WRKY group (Fan et al., 2015).

Several cis-regulatory elements were found in the promoter sequences of NO-responsive AtWRKY TFs at variable distances form the transcriptional initiation site. Mapping of ABRE (TACGTTG), a cis-acting element involved in osmotic stress response and in the regulation of drought response genes; (Kim et al., 2011), EIRE (TTTCGACC, involved in maximal elicitor-mediated activation; (Fukuda, 1997), TATA-box (a core promoter element for enhanced transcription; (Mukumoto et al., 1993), ERE (ATTTCAA, ethylene-responsive elements) (Ohme-Takagi and Shinshi, 1995); MYB binding site (CAACCTG, involved in drought-stress regulation; (Singh and Laxmi, 2015), and both types of W-boxes (TTGACT/C) (Fig. 5), showed NO-induced AtWRKY TFs might regulate the mechanistic control of transcription initiation during abiotic stress or pathogen invasion. Earlier reports claimed that most of the NO-responsive genes had more than one WRKY binding site in their promoter region (Palmieri et al., 2008). Through bioinformatics and functional genomic studies it is now clear that NO-responsive genes contain a significantly higher number of specific transcription factor binding sites (TFBs) in their promoters. Palmieri et al. (2008) analyzed 28,447 Arabidopsis genes and found that several TFBs including several ocs-elements occurred at least 15% more often in NO-induced genes. Of these, the WRKY TF binding sites occurred at least 29% more often in the up-regulated NO-induced genes than in the down-regulated genes, whereas, 17% more often than in control plants.

Furthermore, we compared the expression patterns of the DEGs encoding WRKY TFs with those of other reports in response to different stimuli (Table 1). Interestingly our results were strongly correlated to micro analysis performed in response to ozone treatment. Treatment of Arabidopsis plants with air containing 350 nL L−1 ozone induced the production of NO in plants within 1.5 h of the exposure. NO production was first detected in the guard cells which then spread to the epidermal cells within a 3 h period and ultimately to the mesophyll cells within 8 h indicating a faster O3− induced − NO production in plant tissues. CySNO is a low molecular weight NO donor which is readily absorbed into the plant tissues and has a faster NO release rate (or may be similar to that of ozone) which may possibly be the reason for highly similar effects on gene expression.

The RNA-Seq transcriptome data was validated by qRT-PCR analysis for 16 NO-responsive AtWRKYs involved in different processes, including AtWRKY48, involved in plant defense (Xing et al., 2008), AtWRKY33, involved in abiotic stress regulation (Davletova et al., 2005), AtWRKY62, involved in ABA signaling (Ren et al., 2010), AtWRKY47, involved in jasmonic acid signaling (Schuttenhofer et al., 2014), and AtWRKY22, involved in leaf senescence (Zhou et al., 2011). The high consistency between RNA-Seq and qRT-PCR analysis, supported by a high correlation coefficient (Crombach’s alpha = 0.857), indicated the high reliability of our RNA-seq analysis (Fig. 7). A model representing NO-responsive AtWRKY TFs suggesting significant changes in genes transcription after increase in cellular NO levels. Thus NO-induced signal transduction may trigger changes in TFs regulating transcription of genes putatively involved in plant defense, abiotic stress responses, hormone signaling and growth and development (Fig. 7). The classification of the WRKYs in Fig. 7 is based on TAIR description (https://www.arabidopsis.org/) as the exact functions of most of the WRKY genes has not been experimentally confirmed and is mainly based upon GO enrichment analysis (or TAIR description).

Our RNA-seq transcriptome shows that among total WRKY TFs in Arabidopsis (72), only 33 responded to CySNO (about 45.8%) suggesting that NO regulates different biological processes. We further investigated differences in residues and structures of NO-responsive and non-responsive WRKYs and found that the selected NO-responsive WRKYs have either tyrosine (T) or cysteine (C) residue while the non-responsive WRKYs don’t have the fore-mentioned residues. These two sites (Y and C) may be potential targets for tyrosine nitration and S-nitrosylation, respectively as suggested by earlier reports (Wang et al., 2009; Zaffagnini et al., 2013). Fig. 6 shows the difference of amino acid sequence between NO-responsive and NO-non responsive WRKY TFs. We found that NO-responsive WRKYs have either Y or C residue at the seventh position after the C-terminal WRKYGQK motif while non-responsive one does not have that residues. Both Y and C or known residues for potential targets of protein S-nitrosylation and tyrosine nitration (Sharma et al., 2016). Nitric Oxide is well-known to Post-
translationally modify various proteins by reversibly binding to Cysteine and/or Tyrosine amino acids (process known as S-nitrosylation and/or Tyrosine nitration, respectively). S-nitrosylation of proteins is considered as most important mechanism to transduce the bioactivity of NO in plants (Mengel et al., 2013). Furthermore, this protein modification can modify the expression patterns of downstream genes for example, the transcription factor OxyR regulates bacterial antioxidant genes expression via redox modification of a single cysteine residue (C199) (Hausladen et al., 1996). Nitrosylation (S-NO) of this thiol induces expression of antioxidant genes that are dependent upon cysteine modification. Therefore, changes in the amino-acid sequence can significantly alter the expression of downstream genes in NO signaling cascade. We therefore, discussed the possibility and functional outcomes of the NO-responsive WRKYs being S-nitrosylated, as the list of NO-responsive TFs originally came from a transcriptomic study performed after exogenous NO application and only these genes contained Cysteine and Tyrosine residues.

Previous studies identified the expression patterns of a few WRKY TFs in response to NO (Palmieri et al., 2008) and characterized it in different species, such as cotton (Ding et al., 2015), castor bean (Zou et al., 2016), strawberry (Zhou et al., 2016), rapeseed (He et al., 2016), and Arabidopsis sp. (Eulgem and Somssich, 2007). However, the biological functions and characterization of most AtWRKY TFs are still unknown. In the present study, we performed the first transcriptome-wide characterization of NO-responsive members within the WRKY family in A. thaliana based on high throughput transcriptome data. As WRKY TFs are unique to plants, studying NO-induced WRKY TFs will portray a clear picture of the role of NO in the transcriptional control of different plant genes (Fig. 8).

**Conflict of interest**

The authors declare no conflicts of interests.

**Authors contributions**

QMI, A.H, and B-WY designed the study, QMI, B-GM and SUL, performed experiments, HK, RNK. AA and SA analyzed the data, QMI and A.H wrote the manuscript, IL and B-WY supervised and critically reviewed the manuscript.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2018.01.010.

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