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Transcriptomic Analyses on the Role of Nitric Oxide in Plant Disease Resistance

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Abstract
Nitric oxide (NO) is a gaseous molecule having key roles in many physiological processes such as germination, growth, development and senescence. It has been also shown the important role of NO as a signaling molecule in the response to a wide variety of stress situations, including both biotic and abiotic stress conditions. In the last few years, a growing number of studies have focused on NO-cell targets by several approaches such as transcriptomic and proteomic analyses. This review is centered on offering an update about the principal medium- and large-scale transcriptomic analyses performed with several NO donors including microarray, cDNA-amplification fragment length polymorphism (AFLP) and high throughput sequencing (RNA-seq technology) approaches mainly focused on the role of this reactive nitrogen species in relation to plant disease resistance. Different putative NO-responsive genes have been identified in different plant tissues and plant species by application of several NO donors suggesting the implication of NO-responsive genes with plant adaptive responses to biotic stress processes. Finally, it is also provided an overview about common transcription factor-binding sites of NO-responsive genes and the need to further analyze the different NO-targets by other omics studies.

Introduction
Nitric oxide (NO) is a small and gaseous molecule reportedly involved in a wide array of functions in vegetal systems. In this sense, NO has been shown to be related to defense response (Delledonne et al., 1998; Durner et al., 1998), growth and development (Beligni and Lamattina, 2000; Pagnussat et al., 2002), senescence (Begara-Morales et al., 2013; Beligni and Lamattina, 2001) and in the response to abiotic (Begara-Morales et al., 2014a; Corpas and Barroso, 2013; Corpas et al., 2008; Chaki et al., 2011a; Chaki et al., 2011b; Valderrama et al., 2007) and biotic stress conditions (Feechan et al., 2005; Lindermayr et al., 2005). NO and NO-derived molecules are referred as reactive nitrogen species (RNS) and among them are included peroxynitrite ($ONOO^-$) or nitrogen dioxide (NO_2^-). These RNS are involved in different stressful situations since they can mediate several post-translational modifications through mechanisms such as *S*-nitrosylation and protein tyrosine nitration (Begara-Morales et al., 2013; Corpas and Barroso, 2013; Chaki et al., 2009; Lozano-Juste et al., 2011; Valderrama et al., 2007). Furthermore, it has been demonstrated that NO is important in plant-pathogen interaction being involved in disease resistance (Huang et al., 2002) and having shown that this RNS is quickly produced after pathogen attack and being involved in cell signaling during hypersensitive response (HR) in plants (Delledonne, 2005).

Otherwise, pathogen recognition triggers a rapid production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) or superoxide (O_2^-) (Lamb and Dixon, 1997). This oxidative burst promotes several defense mechanisms like cross-linking of the cell wall (Bradley et al., 1992) and the expression of genes involved in cellular protection and defense (Levine et al., 1994). In this regard, it has been shown that ROS are necessary for the initiation of hypersensitive response (HR) and thereby limiting damage of the infection site (Lamb and Dixon, 1997). Furthermore, it has been demonstrated that a synergistic production of ROS and NO potentiates the induction of hypersensitive cell death in soybean cells therefore showing that NO plays a key role in disease resistance in plants (Delledonne et al., 1998; Delledonne et al., 2001). Accordingly, activation of immune responses in plants constitutes an imbricated response associated with a parallel burst of both ROS and NO.

In recent years, significant advances have been achieved in understanding the molecular basis of the role of nitric oxide in plant-pathogen interactions and in disease resistance. For this proposal, it has been carried out several transcriptomic studies by medium- and large-scale approaches including cDNA-amplification fragment length polymorphism (AFLP) (Ferrarini et al., 2008; Huang et al., 2002; Polverari et al., 2003) and microarray methods (Ahlfors et al., 2009; Badri et al., 2008; Parani et al., 2004; Zeidler et al., 2004) with gaseous NO or different NO-donors. In the last few years, new high-throughput sequencing methods, called massively parallel sequencing or RNA-seq, have emerged as a useful tool that could replace and improve upon existing methods given their advantages as compared to array-based methods (Van Verk et al., 2013; Wilhelm and Landry, 2009). In this regard, it has recently analyzed the gene expression profile by RNA-seq technology of *Arabidopsis thaliana* plants

incubated with S-nitrosoglutathione -GSNO- (Begara-Morales et al., 2014b) which is considered a biological reservoir of NO both in animal (Zhang and Hogg, 2004) and vegetal systems (Wang et al., 2006).

In this review, we summarize recent results obtained by different transcriptomic approaches to update the understanding of NO-responsive genes involved in plant disease resistance. Furthermore, we propose other "omic" experiments to complete and well-define the results obtained by these transcriptional studies to improve our knowledge in the imbricated network in which NO is involved.

Nitric oxide in physiological and abiotic stress processes

Several studies about the role of nitric oxide in plant physiology have been carried out. In this regard, it is well known that NO has an important role in plant growth and development (Corpas et al., 2006; Ribeiro Jr et al., 1999) due to it is involved in different physiological processes such as germination and alleviation of seed dormancy (Beligni and Lamattina, 2000; Bethke et al., 2007; Bethke et al., 2006; Libourel et al., 2006; Zheng et al., 2009). Furthermore, this RNS is implicated in the regulation of plant metabolism and senescence (Begara-Morales et al., 2013; Guo and Crawford, 2005; Leshem et al., 1998), promoting cell death (Pedroso and Durzan, 2000), regulating stomatal movement (Garcia-Mata and Lamattina, 2007) and photosynthesis (Takahashi and Yamasaki, 2002), gravitropism (Hu et al., 2005), regulation of flowering time (He et al., 2004), flower development and apical dominance (Kwon et al., 2012; Lee et al., 2008) and orientation of the pollen tube growth (Prado et al., 2004).

To date, few transcriptional studies have been focused on the role of NO in several physiological aspects. In this sense, the application of different NO-donors like sodium nitroprusside (SNP) in *Arabidopsis thaliana* roots increased the exudation of phytochemicals (Badri et al., 2008). With this respect, several transport systems likely to be involved in the root exudation including MATE, ABC, MFS and other transporters changed their expression in response to NO-treatment. Furthermore and very recently, Monzón et al. (2014) (Monzón et al., 2014) have been determined by microarray technology that NO is required for regulating root architecture and lignin composition in sunflower. In this study, the application of cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tretramethylimidazoline-1-oxyl-3-oxide), a NO scavenger, showed that a fine adjustment of NO levels in the roots would be critical to regulate lignin composition and therefore branching, root architecture and plant growth. Besides, the role of NO in plant physiology has been also related to plant hormones such as jasmonates or auxins showing an interaction at multiple and diversified levels (Huang et al., 2004; Sanz et al., 2014). In this regard, scarce transcriptional analyses with NO donors have been performed in relation to plant hormone study. One of them consists on a microarray study in *Arabidopsis thaliana* plants with salicylic acid- (SA), methyl jasmonate- (MeJA) and NO-treatments. This research showed a limited overlap at different levels such as in the phenylpropanoid biosynthesis or glucosinolate pathways, being the specific genes different in each treatment (Badri et al., 2008). In sum, there is limited information focused on the role of NO in plant physiology by transcriptional approaches, which could be really interesting to understand the functioning of NO in vegetal systems.

Otherwise, most of the transcriptional studies with NO donors have focused on the involvement of this RNS in relation to biotic stress (Ahlfors et al., 2009; Badri et al., 2008; Ferrarini et al., 2008; Polverari et al., 2003). However, inside these studies there are different descriptions about the participation of NO in different target genes involved in several abiotic stress conditions. In this regard, Parani et al. (2004) (Parani et al., 2004) observed the induction on the transcript levels of several dehydration responsive element-binding (DREB1 and DREB2) and late embryogenesis abundant (LEA) proteins by a NO donor as is SNP. The increase on the expression of these genes is related to the tolerance to drought, cold and salinity which could be associated to the drought tolerance observed after NO treatment in wheat (García-Mata and Lamattina, 2001).

Analysis of transcriptomic data about NO-responsive genes related to plant disease resistance

At present, there are scarce transcriptomic studies evidencing the role of NO in plant-pathogen interaction. In this respect, it has been recently performed a differential expression analysis by RNA-seq technology during nodulation of *Medicago truncatula* plants in which NO was removed by incubation with cPTIO (Boscari et al., 2013). This NO depletion provoked a down-regulation in some genes related to nodulation process such as HAP3-like gene, suggesting these results that NO plays a key role in repressing the defense reaction in symbiotic conditions, thereby favoring establishment of the beneficial plant-microbe interaction and differing this action from the signaling function of NO in pathogenic interactions. Moreover, it has been suggested in a recent RNA-seq analysis that in susceptible mutant to *Fusarium* head blight (FHB) disease, NAUH117, both ROS and NO were accumulated while not in resistant mutant (Wangshuibai) variety (Xiao et al., 2013). Authors suggest that this behavior may contribute to the necrotrophic phase during *Fusarium* infection through PCD. Thus, the repression of ROS and NO bursts would lead to enhanced FHB resistance in wheat.

Besides these results, most transcriptional studies that have identified NO-modulated genes were carried out by using pharmacological approaches through the employment of several NO donors like SNP, NOR-3 or GSNO among others. These studies have used medium- and large-scale transcriptional analyses using microarray, cDNA-AFLP and RNA-seq methodologies. First studies were conducted by cDNA microarray containing 200 defense-related genes and 50 genes related to primary metabolism (Huang et al., 2002). Treatment with NOR-3, a NO donor, in *Arabidopsis thaliana* cell suspension cultures highly induced the expression of alternative oxidase 1a (AOX1a), several pathogenesis-related (PR) proteins and a

set of peroxidases and glutathione *S*-transferases. These authors suggest that the induction of AOX1a is SA-independent and that this enzyme might fulfill an important function for cellular homeostasis under NO stress, thereby counteracting the toxicity of NO. Also using microarray technology with 700 defense-related genes, Zeidler et al. (2004) (Zeidler et al., 2004) observed that *Arabidopsis thaliana* leaves react to different lipopolysaccharides (LPS) with a rapid burst of NO and a concomitant activation of defense genes. Among them, they found that LPS induces a broad of defense or stress-associated genes including glutathione *S*-transferases, cytochrome P450 and different PR proteins, altogether contributing to the activation of plant defense responses. Besides these studies, several transcript profiling genome-scale studies were carried out to achieve a global vision of NO-involvement. In this regard, Parani et al. (2004) (Parani et al., 2004) conducted a genome array covering over 24,000 genes in *Arabidopsis thaliana* plants irrigated with 0.1 mM or 1 mM of SNP. This study showed that NO-responsive genes were related to plant defense response, protection against oxidative stress, iron homeostasis, signal transduction and gene expression control through transcription factors such as WRKY members. Moreover, another whole genome microarray with 26,090 genes was used to study and identify root targets modulated by the NO donor SNP in 18-day-old *Arabidopsis thaliana* plants (Badri et al., 2008). In addition to SNP-treatment, it was also performed the incubation with different phytohormones such as methyl jasmonate (MeJA) and salicylic acid (SA) which may control specific signaling pathways different from NO-treatment. It was also characteristic that the number of repressed genes was higher than those up-regulated genes by NO and, finally, only a member of NAC transcription factor family, NAC3, seems to be regulated by the three signaling compounds analyzed. Following with whole-genome microarray analysis, an SNP-treatment of 21-day-old *Arabidopsis thaliana* plants provoked the modulation of 614 genes mostly related to phytohormone-treatments and to the response to biotic or abiotic stresses (Ahlfors et al., 2009). Remarkably, plants sprayed with SNP and ozone (O_3) at the same time promoted the expression of a similar subset of genes indicating that both molecules act synergistically. Finally, one of the main findings was that NO appears to attenuate the induction of SA biosynthesis and signaling genes, thereby suggesting that NO could play a role in the initiation of cell death. On the other hand and in addition to microarray studies, changes in NO-responsive genes were also analyzed by cDNA-AFLP transcriptomic profiling. In this regard, it was observed in *Arabidopsis thaliana* leaves infiltrated with SNP the modulation of 120 genes mainly related to signal transduction, defense response and cell death, removal or production of toxic oxygen species, photosynthesis and energy transfer, cellular trafficking and in basic metabolic pathways (Polverari et al., 2003). Moreover, roots from 4-week-old *Medicago truncatula* plants were treated with SNP or GSNO to assess the effect of different reactive nitrogen molecules (Ferrarini et al., 2008) and they were subjected to cDNA-AFLP analysis with a coverage of 55 % of the *Medicago truncatula* transcriptome. Despite SNP and GSNO are NO donors, only 11 % of genes in leaves and 1.6 % in roots exerted the same modulatory effect, suggesting a modulation of gene expression strictly dependent on the form of NO and on the cellular response in different tissues. On the basis of these results, 999 modulated-genes were used to construct a microarray to monitor the expression of NO-responsive genes in this species during an incompatible interaction and during a symbiotic interaction, being 275 and 290 NO-modulated genes, respectively. In the incompatible interaction, several resistance-related genes and those involved in reactive oxygen species generation or in plant tolerance to oxidative stresses, biosynthesis of JA and defense signaling, proteasome degradation pathway and signal transduction cascades were NO-modulated. On the other hand and related to symbiotic interactions, several genes involved in flavonoid biosynthesis, proteasome degradation pathway, redox signaling and reprogramming of plant cell primary metabolism were processes regulated by NO during this symbiotic interaction. Therefore, this study highlights that NO-responsive genes behave differently depending on the plant organ and on the type of interaction and suggest the role of NO in the HR during pathogenic interaction and might regulate important processes of symbiotic nodule development and functioning. Finally, due to the large number of advantages of massively parallel sequencing or RNA-seq, it has been recently carried out a growing number of NO-related studies using this approach. In this regard, Begara-Morales et al. (2014) (Begara-Morales et al., 2014b) have demonstrated the participation of *S*-nitrosoglutathione (GSNO), which is consider as a biological reservoir of NO, in the response to different stress conditions by an RNA-seq analysis. This methodology has a large number of advantages due to it is not necessary to know the transcribed region previously and permit gene expression quantification in a single experiment among other important advantages (Wilhelm and Landry, 2009). Therefore, GSNO-treatment provoked the induction of different heat shock proteins and wound-responsive proteins in *Arabidopsis thaliana* roots probably involved in the response to a wide array of abiotic stress conditions. Furthermore, due to the synergistic production of reactive oxygen species (ROS) during different abiotic stress situations (Apel and Hirt, 2004; Miller et al., 2008), several oxidative stress-related genes were induced by GSNO-treatment such as mitochondria alternative oxidase 3 and 1a, several peroxidases and members of glutathione transferase proteins. Remarkably, the high induction on the expression of methionine sulfoxide reductase b7 gene supports the protection against oxidative damage from RNS by GSNO as it has been shown in other systems like *Escherichia coli* and *Mycobacterium tuberculosis*. In this sense, it is important to note that although most of GSNO-responsive genes have been previously described, they have identified target genes of GSNO such as MSRB7 gene, which had not been previously detected by array-based methods, supporting the advantages of using massively parallel sequencing or RNA-seq. Finally, an important result obtained from this study was the differential gene expression between leaves and roots, suggesting an organ-specific modulation of gene expression by NO. Consequently, this is the first transcriptomic study with a biological NO donor as is

GSNO by a high throughput technology such as RNA-seq methodology. Moreover, Zeng et al. (2014) (Zeng et al., 2014) have analyzed the transcriptional involvement of NO in birch cells (*Betula platyphylla*) by RNA-seq technology. In this regard, NO-treatment identified 403 up- and 971 down-regulated genes after 12 h SNP-treatment which was then confirmed by qRT-PCR. The results of this transcriptome analysis showed that NO is involved in several processes such as protection against ROS by the regulation of key enzymes such as ascorbate peroxidase or different glutathione *S*-transferases, involvement in carbohydrate metabolism and cell wall biosynthesis, terpenoid biosynthesis and growth regulation. Based on these results, authors indicated that birch cell exposure to NO has a significant effect on many key plant processes as it has been previously described for other plant systems.

Promoter analysis of NO-responsive genes

Recent advances had been done about how NO might regulate gene expression. In this sense, several studies have investigated promotor regions of NO-responsive genes. One of these analyses was focused on 28 common up-regulated genes in different experiments with several NO donors like NOR-3 and gaseous NO (Palmieri et al., 2010). In this regard, eight families of transcription factor binding sites (TFBS) occurred at least 15 % more often in the promotor regions of NO-target genes. Among them, authors found the basic region/leucine zipper motif (bZIP) involved in the regulation of many different physiological processes such as biotic and abiotic stress signaling or seed maturation, WRKY family which are members also involved in pathogen defense, senescence and trichome development and MYCL (myelocytomatosis viral oncogene homolog L) transcription factors. These findings suggest an involvement of NO-responsive genes in different processes such as pathways related to the response to biotic or abiotic stress situations and with several physiological processes. Interestingly, NO-treatment promotes the up-regulation of different JA-related genes like 12-oxophytodienoate reductases (OPR1, OPR2 and OPR3) and two lipoxygenases (LOX3 and a putative lipoxygenase protein) suggesting a close connection between NO and JA-associated processes. Although these results should be experimentally validated, they may be a first step to understand the regulatory networks in which NO is involved. Furthermore, Begara-Morales et al. (2014) (Begara-Morales et al., 2014b) have recently observed two core oligomers (AATTAT and AAAACA) found on the promoters of GSNO-inducible genes. Both have been described as GT-1 factor-binding sites in *Phaseolus vulgaris* bean embryo which are located in the upstream promoter region of light-responsive genes. Among the GSNO-inducible promoters and related to AAAACA core oligomer, was found JAZ10 which is a negative regulator of JA signaling whilst, in relation to AATTAT core oligomer, it was related to a DNA-binding transcription factor and with LSU3, a receptor-like kinase. In sum, the most remarkable fact is that a greater part of GSNO-induced genes share these elements on the promoters and therefore this could be a good starting point to understand or to extend our knowledge about GSNO as a signaling molecule.

Concluding remarks

The literature contains numerous studies reporting the effect of exogenously applied NO on plant processes. However, given the diverse nature of NO-functions, it is more informative to perform large-scale gene expression studies in order to understand the signaling networks in which NO is involved. The development of medium- and large-scale transcriptional analysis from a part or whole-genome such as microarray, cDNA-AFLP or RNA-seq, it has become a very useful tool for the study of the role of NO in plant physiology. However, within the existing transcriptional analyses is sometimes difficult to make an efficient comparison due to the different NO donors employed (SNP, SNAP, GSNO, gaseous NO, NOR-3…) and the different model systems studied (*Arabidopsis thaliana*, *Medicago truncatula*, *Glycine max*, *Betula platyphyilla*…) at distinct developmental stages (cell suspension cultures, seedlings, plantlets, leaves, roots…). In this sense and based on the comparison made by Besson-Bard et al. (2009) (Besson-Bard et al., 2009), NO-responsive genes are usually involved in the plant responses to biotic, abiotic stimuli and stresses. Among these genes, they found partially in common several enzymes involved in detoxification of ROS such as different peroxidases and glutathione *S*-transferases, various genes involved in mitochondrial energy linked-processes, tryptophan synthesis and other genes involved in defense response. These data strengthen the emerging picture that NO functions as a signal involved in the plant adaptive response to plant disease resistance. In spite of the advantages of transcriptomic studies, it is important to bear in mind the need to complement them with other "omic" studies like proteomics. In this regard, it is sometimes slightly comparable the induction of gene expression with the induction of protein expression. Furthermore, proteins are really susceptible to NO-post translational modifications (NO-PTM) such as tyrosine nitration or *S*-nitrosylation which could produce conformational changes and therefore affecting the protein activity. For this reason, with the aid of novel techniques such as TMT (tandem mass tags) isobaric labeling coupled to mass spectrometry is possible to identify and quantify protein expression in different conditions (Kozuka-Hata et al., 2013). Among the advantages of this approach are included an increased multiplex relative quantitation, increased sample throughput and fewer missing quantitative values among samples with a higher identification rates which could be very helpful to highlight the role of NO in several aspects like the involvement in different post-translational modifications. Moreover, it is also important to note that transcriptomic studies do not consider three dimensional (3D) structures of proteins which could be a key feature in NO-proteomic studies. In this regard, Chaki et al. (2014) (Chaki et al., 2014) have compared the prediction of candidate proteins to *S*-nitrosylation by several approaches including biotin switch, mass spectrometry and different computer-assisted methods. This study showed that computational analyses are a useful tool for this prediction; however, further development, such as including the three dimensional structure of proteins in such analyses, would improve the identification of *S*-nitrosylation sites. For this reason, the combination of computational prediction and

experimental verification represents a good approach to better understand the molecular mechanisms and the regulatory functions in which NO is involved.

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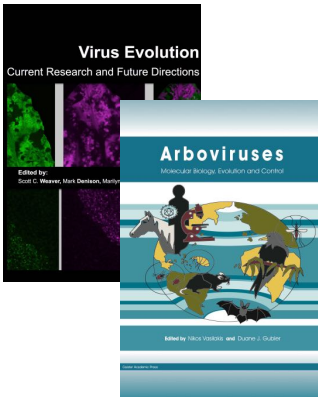

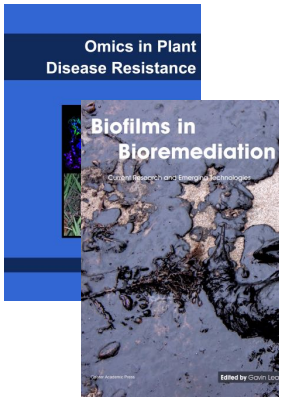

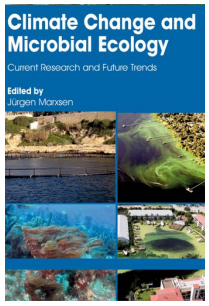

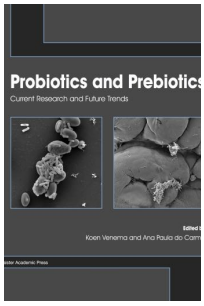

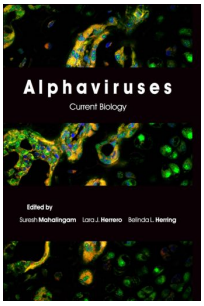

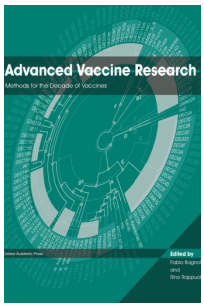

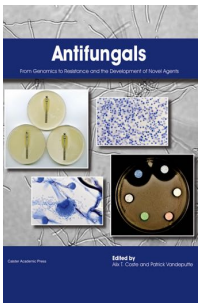

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