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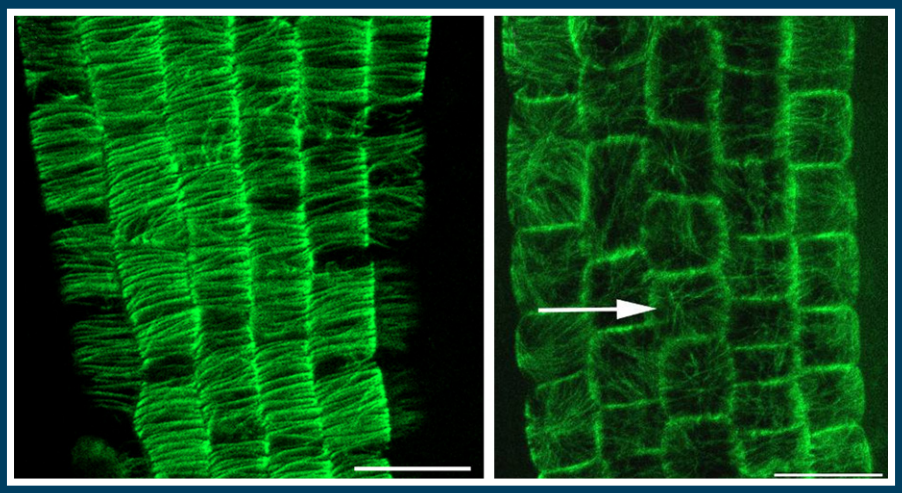

NITRIC OXIDE SIGNALLING IN PLANTS

Topic Editors
Emmanuel Baudouin and John Hancock

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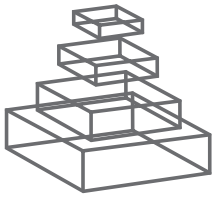

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NITRIC OXIDE SIGNALLING IN PLANTS

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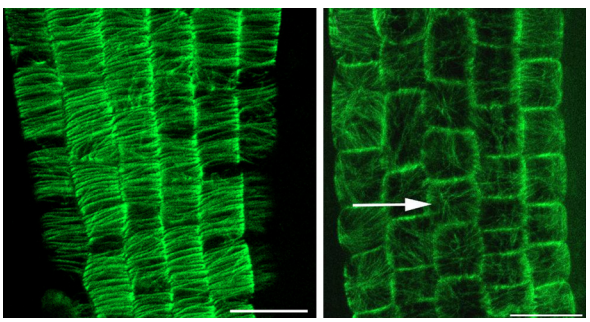

MTs organization in epidermal cells of A. thaliana (GFP–MAP4) primary roots.
Left: Control seedlings (L-Tyr, 200 μM): root apex;
Right: 3-NO2-Tyr (100 μM, 2 h): root apex;

Figure taken from Blume YB, Krasylenko YA, Demchuk OM and Yemets AI (2013). Tubulin tyrosine nitration regulates microtubule organization in plant cells. *Front. Plant Sci.* 4:530. doi: 10.3389/fpls.2013.00530

Nitric oxide (NO) is a free radical ubiquitously participating in plant signalling networks. NO bioactivity has been observed during plant development, from seed germination to flowering and senescence, and in response to most of the environmental cues faced by plants during their lifespan. Although NO is now considered as part of the universal signalling toolbox of plant cells, the sources of NO and how the NO message is converted into a physiological response is still surprisingly obscure. The identification of NO primary targets and NO-regulated genes provides new opportunities to connect NO biochemistry and NO biology. Recent breakthroughs found by comparing NO signalling networks -from the generation of the NO message to its execution into a cellular response- in diverse physiological contexts, opens the way to unravelling how this simple molecule could trigger specific biological outcomes.

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EDITORIAL
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Nitric oxide signaling in plants

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Nitric oxide (NO) is now seen as a vitally important molecule in many biological systems. Once it was identified in mammalian systems in 1987 (Palmer et al., 1987) it was only a matter of time before researchers hunted for its presence in plants, with the first such reports being published in 1998 (Delledonne et al., 1998; Durner et al., 1998). Now 15 years later the interest in NO and its roles in plants is as eagerly investigated as ever, with an ever increasing number of papers published in the area each year. However, the field is not short of controversies, and it would certainly be fair to say that there is still much to be learnt in the area of NO biology. Here, a collection of papers by the many of the most active research groups in the field has been brought together. Some have contributed original research, others have written reviews to enable readers to get a up-to-date view of specialist areas in the field, while others still have written opinion articles, which give their views of the state-of-play in NO research as they see it.

One of the controversies which has caused problems over many years is the way in which NO should be measured in plants. Gupta and Igamberdiev (2013) have contributed an opinion paper and propose that at least two different methods should be used to be sure that NO is truly being measured. This is sound advice and hopefully a strategy that will be adopted by many in the field in the future. D'Alessandro et al. (2013) continue this theme of caution with a paper on the use of cPTIO. This compound is often employed as a scavenger to confirm that NO is being detected, but it is also used as a means to measure the presence of NO when coupled to electron paramagnetic resonance (EPR). These authors report a systematic investigation into the scavenging of cPTIO and discuss the reliability of such use and as an EPR probe.

With the above caveats in place, there seems to be little doubt that NO is present in plants, but a second controversy surrounds the precise sources for its generation. In mammalian systems there are well-characterized nitric oxide synthase (NOS) enzymes but their existence in plants has been thrown into doubt (Hancock, 2012). Correa-Aragunde et al. (2013) compare and discuss the structures of the different NOS enzymes structures across prokaryotes and eukaryotes. In particular they emphasize the presence of such an enzyme in the unicellular microalgae *Ostreococcus tauri*. They do not rule out that higher plants may too have a form of NOS, but only time will tell if they are correct in this opinion. Mur et al. (2013) take a more holistic view of NO metabolism in plants and consider the balance of NO generation, exposure of plants to NO from external sources and the scavenging activity for NO within the plant tissue. They further discuss the impact of the exposure of plant tissues to NOx (NO and NO_2) derived from microbial activity, and ask the question as to whether there is an impact from nitrate metabolism on the overall accumulation of NO in plants.

Since 1998 many studies have shed light on the profound effects NO exerts on plant cell functioning and whole plant development and response to environmental cues. As a first example, Arc et al. (2013) discuss the role of NO in the breaking of seed dormancy and germination. This review presents aspects of NO chemistry in seeds and concludes that NO-dependent protein modification is important during seed germination. Protein modification by NO is a key mechanism when considering downstream effects and is further discussed by others, as described below. As a second example Boscari et al. (2013) address the role of NO in root nodules and in this mini-review question whether NO is actually used as an intermediate in N_2 fixation. Silva and Carvalho (2013) also consider the impact of NO on root nodules, but here the role of glutamine synthetase is the focus, with the suggestion that this enzyme is involved in NO signaling responses in this context. At the other end of the plant, it is also well documented that NO has a pivotal function in the control of stomatal apertures, and this is discussed in a review article by Gayatri et al. (2013), with a particular focus on the interplay between NO and cytosolic pH, reactive oxygen species (ROS) and free calcium ions. The different reviews open routes for future developments in their particular field but all underline that integrating NO into the global redox network is of topical importance. This aspect is specifically discussed in two reviews presented below.

One of the original roles that were determined for NO in plants was in plant defence. In an original article Schlicht and Kombrink (2013) investigate NO function in the defence against fungi. They report the accumulation of NO at infection sites, and suggest that there is a correlation between resistance phenotypes and NO production, both in its timing and accumulation. Groß et al. (2013) further tackle the topic of plant defence by reviewing the interaction of NO with antioxidants and prooxidants. They discuss the reaction of NO with ROS and the formation of other reactive compounds such as peroxinitrite, as well as the removal of NO through the action of non-symbiotic hemoglobins (nsHb). Wang et al. (2013) also consider the role of the interaction of NO and ROS in a review which focuses on the cross-talk between these two important signaling

pathways. Their discussion considers plant defence through the role of NO and ROS on the hypersensitive response, but also on leaf senescence and other types of programmed cell death (PCD).

The intimate relationship between NO and antioxidants is particularly illustrated with the case of glutathione, one of the major antioxidants in cells that is hugely important in the control of ROS metabolism. Indeed, it is well established that there is a key reaction between glutathione and NO which results in the formation of S-nitrosoglutathione (GSNO)—this is the discussion point of an opinion article by Corpas et al. (2013). The authors summarize GSNO metabolism and suggest that investigating GSNO occurrence and function in plant cells, along with other NO reaction products, will be an important issue for the future understanding of the role of NO in plant development and stress responses. Illustrating this opinion the enzymes S-nitrosoglutathione reductases (GSNORs) are the subject of an original article by Xu et al. (2013), where the importance of these enzymes is discussed in view of their role in development and defence. They use bioinformatics and structural modeling to show the location of GSNORs and identify conserved amino acids, which are vital to their role.

The mechanisms that allow NO perception and its conversion into physiological responses have been paid much attention in the last years. In that view proteomics has been used successfully to determine the proteins that may react directly with signaling compounds including NO. Romero-Puertas et al. (2013) consider the formation of the main protein modification that is S-nitrosylation, during abiotic stress. The reaction of NO with protein thiols is an immensely important way in which NO may have its effects in all biological systems, with plants being no exception. París et al. (2013) continue this discussion in a mini review by considering how S-nitrosylation is involved in the workings of hormone networks. Future routes for investigating S-nitrosylation function in plant cells are proposed. Mengel et al. (2013) focus on the role of S-nitrosylation in a particular cell compartment, i.e., the nucleus, and review the effects of NO on gene transcription, with comparison to the work that has been carried out on animal systems. On the other hand, Sehrawat et al. (2013) suggest that the depletion of RuBisCo from samples would be an advantage to broaden the S-nitrosylated protein atlas, and they report on new cold-responsive S-nitrosylated targets in *Brassica juncea*. Another NO-based protein modification is tyrosine nitration and Blume et al. (2013) report the regulation of cytoskeleton organization via the tyrosine nitration of α-tubulin.

How NO, or indeed any other compounds, are able to bring about control of cell function means that there must be an influence on cell signaling events. As can be seen from above NO has an impact on other signaling such as that carried out ROS—as discussed by Wang et al. (2013) for example—as well as having effects on signaling proteins by S-nitrosylation or tyrosine nitration. But other signaling pathways and components can be affected too. Guillas et al. (2013) discuss its impact on sphingolipids, and again use comparisons with the work in animals to aid in the understanding of the way such molecules interact in plants. They conclude that although the generic idea may be common across eukaryotes the details will be different. On the other hand in a mini review Salmi et al. (2013) consider how NO may mediate the signaling by extracellular nucleotides. Such extracellular signals have been found to induce NO generation in plant cells, and this paper discusses this with particular reference to auxin signaling and plant growth. This underlines the crucial role of NO in the control of events initiated by phytohormones that is the subject of a review by Freschi (2013). Here the focus is on the pathways that regulate metabolism and development but this paper also brings together many elements of NO signaling discussed by others in this collection, including gene expression, defence responses, and post-translational protein modifications.

The field of NO generation and effects in plants has moved a long way since it was first suggested in 1998, and this collection of papers discusses many aspects of the area as it stands today. However, it also highlights that there is still a long way to go before there is a clear understanding of how NO is made by plants cells and how NO fits into the signaling that controls so many key aspects of plant growth and development. However, several of the authors have given their opinions and ideas that will be useful to steer the direction of plant NO research in the future.

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OPINION ARTICLE
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Recommendations of using at least two different methods for measuring NO

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INTRODUCTION

Nitric oxide (NO) is widely recognized as a signal molecule in plants. Various sources of NO were identified in plants (Moreau et al., 2010; Gupta et al., 2011; Mur et al., 2013) located in different compartments activated under various conditions. Briefly these are the mitochondrial nitrite: NO reductase reaction, the cytosolic nitrate reductase (NR), the plasma membrane nitrite: NO reductase (PM-NiNOR), xanthine oxidoreductase, NO synthase-like enzyme (putative), polyamine (PA)- and hydroxylamine (HA)-mediated pathways. NO acts as an intracellular messenger due to its diffusible capacity through various cellular compartments. After pioneering discovery of NO production in plants, scientists started digging deeply to the key function of NO and this research led to understanding of various roles of NO that include regulation of stomatal movement, root development, floral transition, response to biotic and abiotic stresses, symbiotic interactions. Despite of extensive research on NO roles in metabolism and signal transduction, its measurement remains challenging (Vandelle and Delledonne, 2008; Mur et al., 2011). We discuss below the problems associated with NO measurement and suggest some important solutions to tackle these problems.

The half-life of NO depends on its concentration and usually falls is in the range of ten(s) seconds. For instance, at 10 μM concentration NO has a half-life of about 80 s whereas at 100 μM concentration NO has a half-life of about 8 s (Wink and Mitchell, 1998). This means that at low concentration NO can easily diffuse from its origin to the site of action. At higher concentrations most of the NO rapidly undergoes autoxidation. Moreover NO half-life also depends on the presence of scavengers such as non-symbiotic hemoglobin (class 1) and other major NO scavenging targets such as lipids and metal-containing proteins. Therefore, it is often very important to measure NO concentration which is crucial for understanding its function. But NO concentrations measured in same biological material by different methods often give different values (Mur et al., 2006; Planchet and Kaiser, 2006). In some cases NO production is localized to specific cells such as guard cells; therefore fluorescent probes are required to visualize NO producing sites. Here we describe why we need to use at least two different methods for measuring generation of NO in plants and suggest the best combination of methods for specific studies.

OVERVIEW OF NO DETECTION METHODS: ADVANTAGES AND TECHNICAL PROBLEMS

MEASUREMENT OF NO IN THE GAS PHASE

Chemiluminescence is a well-established method for NO measurement. In this method the reaction between NO and ozone (O_3) generates nitrogen dioxide (NO_2^*) in the excited-state which then emits a photon and reaches its ground state, a photomultiplier counts the light generated in the amount proportional to NO content. Chemiluminescence has been used to measure NO emissions from leaves, roots and from isolated mitochondria (Planchet et al., 2005; Gupta et al., 2011; Shah et al., 2013). This method is highly sensitive and can detect NO in the range of parts per billion which corresponds to picomolar concentrations in the tissue. The major disadvantage of this method is that it measures only the emitted NO in the gas phase (Planchet and Kaiser, 2006). Another disadvantage is that it measures only pure NO emitted from biological samples, while only a small portion (in green leaves of Arabidopsis less than 6%) of the produced NO is emitted from the biological samples, the major part is quenched in the reaction with superoxide (Vanin et al., 2004), and in the hypoxic tissues scavenged by the non-symbiotic hemoglobin (Igamberdiev et al., 2006).

Laser-based photoacoustic detection of NO uses the absorption of rapidly chopped infrared light by NO (Mur et al., 2011). The sound is generated during the absorption and relaxation which is detected by the microphone located in the photoacoustic cell. This method was used by Mur et al. (2005) to detect NO from tobacco leaves infected with bacteria (Pseudomonas). The advantage of this method is it very high precision, while the disadvantage is that NO can be detectable only in the gas phase and no special information about NO production in specific cells can be obtained. This method should be used together with DAF fluorescence (see below) to get the information about the presence of NO in the gas phase and in specific cells respectively.

Quantum cascade laser-based spectroscopic detection of NO (Moeskops et al., 2006; Gupta et al., 2013) represents another version of the previous method. The laser is integrated with a thermo-electrically cooled infrared detector. The detection sensitivity of this method is 0.03 parts per billion which is higher by 1–2 orders of magnitude than in the photoacoustic detection.

The Membrane Inlet Mass Spectrometry (MIMS) is a robust method for NO detection (Conrath et al., 2004). In this method the online detection of NO in gaseous phase is possible. The membrane barrier separates the sample from mass spectrometer and allows NO to be detected. Another advantage of MIMS is that, by using radioactive substrates, it is possible to detect the contribution of NO from each pathway by using radiolabelled arginine or nitrate/nitrite (Conrath et al., 2004). Though it is an excellent and very sensitive method, it was rarely used in plant NO research. This is probably due to its high cost and expertise requirement. In this method, as in other gas phase methods, the detection of oxidized forms of NO is not possible. But after measuring NO by this method, the samples can be ground and further analyzed by using mass spectrometer, indirect chemiluminescence, Griess reagent or NO electrodes.

MEASUREMENT OF NO IN THE LIQUID PHASE

A common method of measuring NO in liquid phase is based on using the NO electrodes. These include platinum/teflon or platinum/iridium (Pt/Ir) coated working electrode and Ag/AgCl reference electrode. NO is detected via its oxidation at +0.8 to +0.9 V compared to the reference electrode (Shibuki, 1990). NO electrodes have been used in plant NO research, e.g., to measure NO production in tobacco cells in response to cryptogein (Besson-Bard et al., 2008), to detect NO in fruits (Leshem, 1996), to measure NADH-dependent NO scavenging activity in plant extracts and in purified fractions containing class 1 hemoglobin (Igamberdiev et al., 2004, 2006). The disadvantage of this method is that it measures NO in the liquid phase and if plant tissues emit NO in the gaseous phase, it is not quite reliable to detect it using these electrodes. The best combination is the use of the chemiluminescence method to detect NO in the gas phase and then NO electrodes to check NO concentration in the extracts of plant tissues.

As we have mentioned above, NO is highly reactive and only its small portion is emitted from the biological samples while the rest is oxidized (Vanin et al., 2004), therefore it is not possible to measure oxidized forms of NO such as nitrate, nitrite by gas phase chemiluminescence. But this limitation can be overcome by using indirect chemiluminescence in which nitrate and nitrite produced from oxidation of NO are reduced back to NO by injecting sample extracts into boiling acidic vanadium chloride (Gupta and Kaiser, 2010).

If there is no proper equipment for doing indirect chemiluminescence, then the alternative method to measure oxidized forms of NO is the Griess reagent assay, which is relatively cheap. In this method NO is oxidized to nitrite which reacts with sulphanilic acid and α-naphthylamine under acidic conditions to produce the azodye which can be detected at 520 nm. This method is not commonly used by plant scientists but there are few reports (Shirinova et al., 1993; Planchet et al., 2005). Sensitivity is very low for this method (0.5 μM). Vitecek et al. (2008) showed that by using two traps (one for gas phase and one for liquid phase) it is possible to measure NO both is gas and liquid phases. The main problem of this method is the interference of internal nitrite which concentrations are much higher than NO.

Electron-spin resonance (ESR) is a well-accepted method to detect NO in a liquid phase. This method is based on detection of unpaired electrons that exhibit resonance in opposite orientations. This method is very specific for NO detection and its limit is in the picomolar range (Weaver et al., 2005). But the ESR spectrometer is expensive and special expertise is needed for operation. It cannot measure the emitted NO but it rather detects the trapped NO, and the online measurement of NO is not possible by this method.

Another biochemical assay to measure NO is the oxyhemoglobin assay. It is based on the reaction of oxyhemoglobin (HbO_2) with NO resulting in the production of methemoglobin (MetHb) and nitrate (NO_3^-). Methemoglobin is detected at 401 nm. This method has considerable sensitivity which is in the nanomolar range (Murphy and Noack, 1994). This assay has been used by plant scientists (Cvetkovska and Vanlerberghe, 2012). Although it has a good sensitivity, the serious problem is that the reactive oxygen species oxidize HbO_2 and give false positive results. The changes in pH can also affect the reaction. Since both ROS production and pH change are a part of stress response, caution should be taken while interpreting the results used via this method. NO is a free radical molecule that escapes from the site of production to target and also diffuses to the atmosphere. By the time hemoglobin assays is done there is a huge possibility that NO escapes from the sample. The techniques like oxyhemoglobin assay should be coupled with another measurement method like chemiluminescence, which can measure NO in the gas phase.

A widely used and most controversial method for NO detection is diaminofluorescein (DAF) fluorescent dyes (Foissner et al., 2000; Lamotte et al., 2004; Corpas et al., 2006; Prats et al., 2008; Cvetkovska and Vanlerberghe, 2012 and many other references). The principle of this method is based on 4,5-diaminofluorescein diacetate (DAF-2DA) diffusion into cells where the acetate groups are removed by intracellular esterases and generate 4,5-diaminofluorescein; DAF-2 can also react with N_2O_3, an oxidation product of NO, to generate the highly fluorescent DAF-2T (triazolofluorescein).

THE USE OF FLUORESCENT DYES: ADVANTAGES AND CAUTIONS

The advantage of DAF dyes that it is very easy to apply them and observe the NO fluorescence using the fluorescent or confocal microscope, which are easily available commercially and cost-effective. If NO is produced in specific sites such as guard cells, meristems or nodules or pathogen-infected cells these dyes can easily react with NO and give good indication about NO production. DAF dyes are relatively sensitive to NO having the detection limit in the nanomolar range and moreover no additional fluorescence is observed with NO_2^-, NO_3^-, H_2O_2, and $ONOO^-$ (Kojima et al., 1998).

However, the application of fluorescent dyes has been challenged by various studies. For instance, DAF2 reacts with dehydroascorbic acid (DHA) and ascorbic acid (AA) and forms fluorescent products within the similar range of fluorescence as DAF-2T (Zhang et al., 2002). Jourd'heuil (2002) was the first who suggested that DAF fluorescence is sensitive to NO only in the presence of superoxide (O_2^-) or peroxynitrite ($ONOO^-$).

This was confirmed in the independent study by Roychowdhury et al. (2002). The value of pH can also affect the fluorescence from DAF (Vitecek et al., 2008). Planchet and Kaiser (2006) compared DAF fluorescence with the chemiluminescence method and found that tobacco cell suspension that produces DAF fluorescence does not necessarily produce NO signal in chemiluminescence. Rümer et al. (2012) have shown that the cyptogein induced tobacco cells produce multiple products that generate DAF-fluorescence *in vitro* which is not attributed to DAF-2T, some of them are attributed to the reaction of apoplastic peroxidase, DAF and H_2O_2 in which DAF was a substrate for peroxidase. Horseradish-peroxidase plus H_2O_2 also generated DAF-fluorescence *in vitro*.

Carboxy-PTIO (cPTIO) is a widely used NO scavenging compound to check whether NO is responsible for the observed fluorescence. The mechanism of cPTIO effect is based on the oxidation of NO to NO_2, and thereby it scavenges NO. But when the excess of NO is produced, NO reacts with NO_2 and forms N_2O_3 which leads to the increased fluorescence. Therefore, the cPTIO-based NO assessment depends on NO concentrations. Moreover Rümer et al. (2012) found that the decrease in DAF fluorescence by applying cPTIO does not necessarily indicate the initial presence of NO since cPTIO can also decrease H_2O_2 production. During various stress conditions plants produce NO and ROS. Therefore, the use of DAF fluorescent dye can hamper the actual situation of NO status. On the other hand, for determing NO production in specific cells such as root tips or stomatal guard cells, fluorescence indicators are necessary to distinguish NO producing cells from non-producing cells. Another frequently used dye is DAF-FMDA having higher sensitivity than DAF-2DA (3 nM). But the disadvantage of DAF-FMDA is that its fluorescence depends on pH (Vitecek et al., 2008). Since plant cells exhibit different pH values at different stress conditions, the DAF-FM method should be used with caution. Another fluorescence dye is diaminorhodamine-4M (DAR-4M) (Lacza et al., 2005) but it is useful for assessment of total reactive nitrogen species (RNS) rather than for measurement of NO.

CONCLUSION

Taken together all the information presented here can teach us various things:

Fluorescent dyes are very useful for detecting NO in specific cells but, as described above, they have various disadvantages. It should be recommended to do independent measurement of DAF-2T using high-pressure liquid chromatography (Rümer et al., 2012). Always one should detect autofluorescence before adding fluorescent dyes to the samples. Only few authors used two methods for NO detection which provides necessary comparison and gives more reliable results. For instance, Planchet and Kaiser (2006) used DAF fluorescence and chemiluminescence; Bright et al. (2009) used DAF fluorescence and EPR; Cantrel et al. (2011) used DAF fluorescence and chemiluminescence assay; Gupta et al. (2013) used chemiluminescence and quantum cascade laser; DAF fluorescence and hemoglobin assay were used by Cvetkovska and Vanlerberghe (2012).

On the other hand, many studies still lacking the practice of using dual approach methods which results in low reliability because of the lack of independent verification of uncertainties generated by the restrictions of one method applied. Therefore, we recommend always using two independent methods with appropriate controls in order to obtain valuable information about NO concentrations and distribution in plant growth, development and stress response. In particular, we recommend using at least one gas phase method (1) and one liquid phase method (2) (See below).

Method 1 (gas phase)	Method 2 (liquid phase)
Chemiluminescence	NO electrodes
Laser-based photoacoustic detection	Indirect chemiluminescence
Quantum cascade laser	Electron spin resonance
Membrane inlet mass spectrometry	Oxyhemoglobin assay
	Griess reagent assay
	Fluorescent based dyes

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Current overview of *S*-nitrosoglutathione (GSNO) in higher plants

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INTRODUCTION

S-nitrosoglutathione is a nitric oxide-derived molecule, generated by the interaction of nitric oxide (NO) with reduced glutathione (GSH) in a process called *S*-nitrosylation (**Figure 1**). The reaction appears to take place either through the formation of N_2O_3 or the addition of NO to a glutathionyl radical formed during this reaction (Broniowska et al., 2013). GSNO is regarded as an intracellular NO reservoir as well as a vehicle of NO throughout the cell, which enables NO biological activity to expand. GSNO is also considered to be the most abundant low-molecular-mass (LMM) *S*-nitrosothiol (SNO). This family includes other molecules such as *S*-nitrosocysteine (CySNO) and *S*-nitrosocysteinylglycine (GlyCySNO), which have been the subject of less study in the field of plant research. There is another group of SNOs called high-molecular mass (HMM) SNOs which are produced by NO binding to sulfhydryl (-SH) groups present in specific cysteine residues of proteins. **Figure 1** shows a simple model of GSNO metabolism and its interactions with other molecules in cells where different reactions including *S*-nitrosylation, *S*-transnitrosation, and *S*-glutathionylation are involved (Hogg, 2002; Martínez-Ruiz and Lamas, 2007). In plants, research has focused on the importance of total SNOs in specific stress situations (Feechan et al., 2005; Chaki et al., 2011a) and on the identification of the potential protein targets of *S*-nitrosylation as this kind of post-translational modification can alter the function of the affected proteins (Astier et al., 2012). Initial studies in this area exogenously applied GSNO in order to identify the pool of potential protein candidates (Lindermayr et al., 2005). However, less attention has been paid to the abundance, distribution, and modulation of endogenous GSNO under natural and stress conditions. In this article, we will provide a current overview of GSNO in higher plants.

DETECTION AND QUANTIFICATION OF GSNO IN PLANTS

Although, a considerable number of studies of NO in plants are available, much less information exists regarding SNOs and, more specifically, GSNO. This is mainly explained by the fact that the determination of GSNO in plant samples still presents a challenge in analytical terms due to several technical obstacles and the often lengthy sample preparation procedures required. In addition, other potential problems are caused by the intrinsic instability of GSNO in plant samples. Thus, the determination of GSNO can be affected by light, metal-catalyzed GSNO decomposition, enzymatic degradation as a result of endogenous GSNO reductase activity and a reduction in the *S*-NO bond caused by reductants and endogenous thiols.

In higher plants, two different approaches to detect GSNO have been reported: immunohistochemical analysis using commercial antibodies against GSNO (Barroso et al., 2006; Valderrama et al., 2007) and liquid chromatography-electrospray/mass spectrometry (LC-ES/MS) (Airaki et al., 2011). These techniques have provided some initial background data on cell localization in different organs and on GSNO content under development and adverse stress conditions. Whereas immunohistochemical localization using fluorescence probe as secondary antibody can provide localization a relative abundance with high sensitivity, LC-ES/MS is the technique that provides a most consistent quantification. The reported GSNO content ranges between 3 and 8 nmol GSNO g^{-1} fresh weight (Airaki et al., 2011) which is in the same range of oxidized glutathione (GSSG).

FUNCTION OF GSNO UNDER ADVERSE ENVIRONMENTAL CONDITIONS

At present, some data shows that GSNO is an important molecule in the mechanisms of response to biotic and abiotic stress. Immunohistochemical analysis using confocal laser scanning microscope (CLSM) in several plant species under different stress conditions has enabled the spatial and relative content of GSNO to be determined. In pea plants, the content of GSNO localized in leaf collenchyma cells and under 50 μM cadmium stress was drastically reduced, which was accompanied by a 31% reduction in GSNOR activity (Barroso et al., 2006). In addition, *Arabidopsis thaliana* exposed to a toxic concentration of arsenic causing nitro-oxidative stress showed a significant reduction in GSNO content detected by LC-ES/MS. However, GSNOR activity, which increased significantly, showed an opposite tendency (Leterrier et al., 2012). In the case of olive plants grown in the presence of 200 mM NaCl, the localization and relative GSNO content evaluated by CLSM were totally different, with salinity causing a marked increase in GSNO activity, mainly in the vascular tissue (Valderrama et al., 2007).

In sunflower plants, GSNO has been studied under biotic and abiotic stresses. CLSM analysis of hypocotyl sections of plants exposed to abiotic stress (mechanical wounding and high temperatures) showed a general accumulation of GSNO

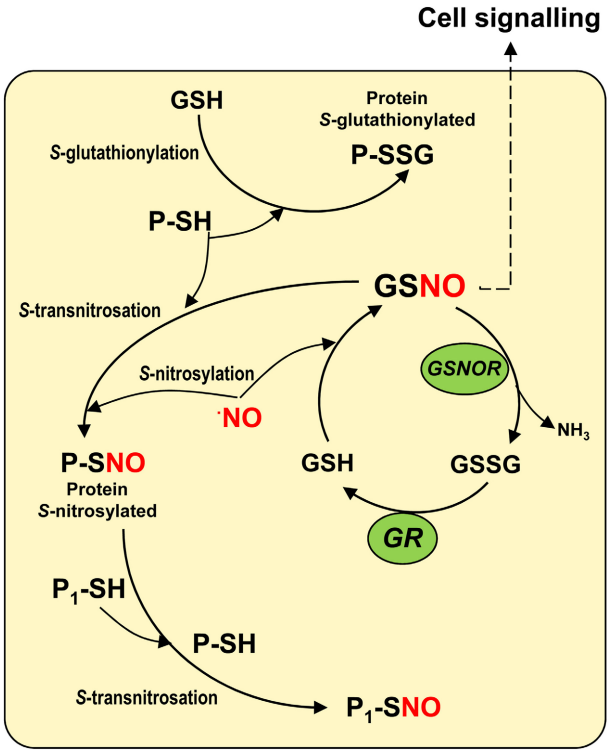

FIGURE 1 | Model of the *S*-nitroglutathione (GSNO) metabolism in cells. The interaction between reduced glutathione (GSH) and nitric oxide (NO) enables GSNO to be generated by a process of *S*-nitrosylation. GSNO could be decomposed by the GSNO reductase to oxidized glutathione (GSSG) which is the substrate of the glutathione reductase (GR) that regenerates the reduced glutathione. GSNO, regarded as the most abundant low-molecular mass (LMM) *S*-nitrosothiol, can interact with specific sulfhydryl (-SH) groups of proteins to produce high-molecular mass (HMM) *S*-nitrosothiols in a process called *S*-transnitrosation (Hogg, 2002). HMM S-nitrosothiols can also transfer NO to the sulfhydryl (-SH) groups of other proteins (P_1-SH) through a process of *S*-transnitrosation between proteins. On the other hand, GSH can interact with specific sulfhydryl (-SH) groups of proteins in a process known as *S*-glutathionylation.

in all hypocotyl cells, with a concomitant reduction in GSNOR activity, thus mediating nitrosative stress (Chaki et al., 2011a,b). Similar behavior was observed in sunflower under biotic stress, specifically in relation to the fungus *Plasmopara halstedii*. However, it is interesting to note that GSNO was observed to be localized and distributed in the sunflower hypocotyls of the resistant cultivar, while GSNO showed a general and homogenous distribution in all hypocotyl cell types. This appears to contribute to its resistance to fungus, with GSNO after infection being exclusively redistributed to the epidermal cells which are usually this pathogen's penetration sites in sunflowers (Chaki et al., 2009). GSNO mobilization has also been described in wounded *Arabidopsis* leaves where GSNO content increased and showed a uniform distribution pattern, whereas, in systemic leaves, GSNO was first detected in vascular tissues and later extended to the parenchyma cells (Espunya et al., 2012). These findings in relation to different plant species and under different stresses bolster the notion that GSNO appears to be a mobile signal in response to diverse types of stress. Although, the experimental evidence suggests the GSNO movement between plant cells and organs, future specific experiments will be needed to confirm it.

S-NITROSOGLUTATHIONE AND PLANT DEVELOPMENT

The effect of NO on seed germination, root architecture, development, and fruit ripening has been routinely studied using NO donors such as sodium nitroprusside. However, more recently, GSNO has begun to be used as it is considered to be a more physiological NO donor (Liu et al., 2007; Zandonadi et al., 2010; Semchuk et al., 2011). However, to our knowledge, there is no information on the content of endogenous GSNO during these plant processes. As mentioned earlier, the use of LC-ES/MS to detect and quantify GSNO has provided some initial data on GSNO content in plant organs. Thus, analysis of GSNO in the main organs of pepper plants have indicated that GSNO was most abundant in roots, followed by leaves and stems, which directly correlated with the content of NO in each organ and inversely correlated with GSNOR activity (Airaki et al., 2011). Very recently, it has been also reported the subcellular localization of GSNO in pea leaves by electron microscopy immunocytochemistry and immunogold particles were clearly visible in cytosol, chloroplasts, mitochondria, and peroxisomes (Barroso et al., 2013).

On the other hand, the involvement of NO in plant reproductive biology has been reported (Bright et al., 2009; Zafra et al., 2010). Thus, NO can act as a negative regulator of pollen tube growth in plants such as *Lilium longiflorum*, *Arabidopsis thaliana*, and *Paulownia tomentosa* (Prado et al., 2004, 2008; He et al., 2007) and as a positive stimulus of pollen tube growth in *Pinus bangeana* in a dose-dependent manner (Wang et al., 2009). Recently, analysis of GSNO by LC-ES/MS in olive pollen subjected to *in vitro* germination has shown the presence of GSNO (unpublished data) whose content closely correlated with the NO content previously reported (Zafra et al., 2010) and inversely correlated with *GSNOR* gene expression.

CONCLUSIONS

The study of GSNO, which is part of the metabolism of NO in higher plants, has begun to increase our knowledge of the physiological significance of this universal molecule that is involved in almost all the process where GSNO has been studied. Consequently, the analysis of GSNO content and metabolism during plant development and under environmental stress conditions presents a new challenge in relation to the signaling properties of GSNO.

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Structure diversity of nitric oxide synthases (NOS): the emergence of new forms in photosynthetic organisms

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NO SYNTHESIS FROM MAMMALIAN AND PHOTOSYNTHETIC ORGANISMS

Humans have enormously increased the level of nitrogen (N) circulating in the troposphere and the earth surface during the last century, correlating with the population increase. As an undesirable consequence, high levels of reactive N are polluting the environment where humans inhabit. Nitric oxide (NO) is one of the reactive N species with both positive and negative impact on life. NO synthases (NOSs) are enzymes that oxidize arginine to citrulline and generate the denitrifying intermediate NO which can be subsequently reduced to N_2O and N_2. NOS are large modular enzymes present in all kingdoms which through evolution were the result of multiple gene and genome duplication events together with changes in protein architecture (Andreakis et al., 2011). A recently described NOS from the marine unicellular microalgae *Ostreococcus tauri*, belonging to the picoplankton in oceans, adds new insights to study the evolution of the complex organization of these enzymes. In this opinion we discuss the structure diversity of the emerging new NOS forms described in prokaryotes and eukaryotes. Regarding the controversy about the existence of canonical NOS in higher plants, we propose that the latest findings support the existence of a high diversity of NOS forms in different lineages. Thereby, since higher plant species whose genomes have been fully sequenced, which are scarce, it cannot be discarded that a new form of NOS may have evolved in higher plants.

Mammalian NO synthases (NOSs) were the first NOS structures to be biochemically characterized, crystallized and their complete structure deciphered by X-ray diffraction. These NOSs are functional as homodimers, each monomer consisting on an N-terminal oxygenase domain (NOSoxy) containing the binding sites for the cofactors heme, tetrahydrobiopterin (BH4) and for the substrate L-arginine, and a C-terminal reductase domain (NOSred) that binds to NADPH, FAD and FMN (**Figure 1**). Mammalian NOSs require the binding of calmodulin (CaM) for electron transfer from reductase to oxygenase domain. NOSred has strong sequence similarity with NADPH cytochrome P450 reductase (Stuehr, 1999; Alderton et al., 2001). The crystal structure of NOS showed that there is a zinc tetrathiolate center in the dimeric form of NOS. The zinc ion is coordinated by 4 Cys, two from each subunit. The Cys-X_4-Cys motif involved in Zn coordination seems to be conserved in all animal NOS described so far (**Figure 1**). The NOSoxy and NOSred domains have been separately cloned and expressed as recombinant proteins without alteration of their catalytic properties (McMillan and Masters, 1995; Chen et al., 1996). Particularly in mammals, there are three distinct NOS isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) encoded by three different genes. These three isoforms differ in localization, regulation and catalytic properties (Alderton et al., 2001). The isolation and characterization of NOS proteins from different species from different kingdoms indicate that NOSs differ structurally and biochemically. Moreover, it has been suggested that NOS could catalyze different reactions depending on substrate and cofactors concentrations (Weaver et al., 2005).

The recent identification of the NOS from the green algae *Ostreococcus tauri* represents the first NOS characterized in photosynthetic organisms (plant kingdom) (Foresi et al., 2010). Ostreococcus NOS (OtNOS) has a 42% of similarity to human NOS reaching to 45–49% similarity to invertebrate NOS. OtNOS contains the NOSoxy and NOSred domains joined by a CaM binding domain (**Figure 1**). Despite the high similarity, some differences could be noted in the structure of the OtNOS with respect to animal NOS. CaM plays a critical role in activating NOS, since it triggers the electron transfer from flavin to heme. In eNOS and nNOS the electron transfer is triggered by CaM binding while in iNOS, CaM is irreversibly bound. That explains why iNOS is active independently of Ca^{2+} concentration. Indeed OtNOS activity behaves like an intermediate between eNOS/nNOS and iNOS isoforms since in the absence of Ca^{2+}-CaM, OtNOS retains almost 70% of activity. Furthermore, OtNOS lacks of the autoregulatory control element (ACE) (Foresi et al., 2010), indicating that it is close to the mammalian iNOS isoform. The ACE impedes CaM binding and enzymatic activation in constitutive NOSs. The increase in Ca^{2+} concentration triggers the binding of Ca^{2+}-CaM in constitutive NOS by displacing the ACE (Salerno et al., 1997). The Zn binding motif Cys-X_3-Cys in OtNOS is partially conserved compared to Cys-X_4-Cys in mammalian NOS. Even though the binding of Zn to OtNOS has not been experimentally proved, there are other examples of Zn binding motif consisting of Cys-X_3-Cys (Barbosa et al., 1989; Vasak and Hasler, 2000). BH_4 cofactor is essential for NO production in animals since the absence of BH_4 uncouples the reaction leading to NADPH oxidation and superoxide formation. Ostreococcus genome has been completely sequenced (Derelle et al., 2006) and it lacks the genes encoding for the enzymes that synthesize BH4, suggesting that OtNOS may bind another cofactor for catalytic activity.

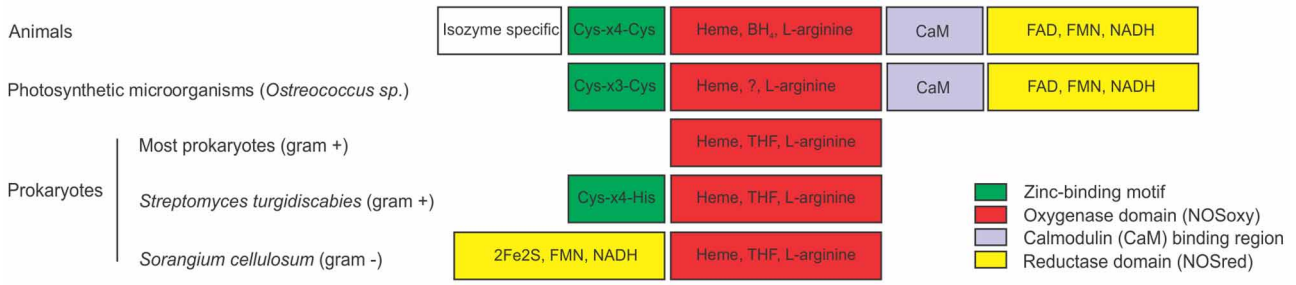

FIGURE 1 | Structures of nitric oxide synthases (NOSs) from different sources. Comparison of animal NOS structure with NOSs from photosynthetic microorganisms and prokaryotes. Animal NOSs contain a zinc-binding region (Cys-X4-Cys), a NOS oxygenase domain (NOSoxy) which binds Heme, arginine and BH_4, a calmodulin-binding region (CaM) and a NOS reductase domain (NOSred), which binds FMN, FAD, and NAD. The only described NOS of the photosynthetic organism is from the Ostreococcus genus. It has the NOSoxy, NOSred, a partially conserved CaM domain and a Zn-binding motif that partially differs from animals (Cys-X3-Cys). Most prokaryotes has only the NOSoxy domain, with the exception for the gram negative bacterium *Sorangium cellullosum* that has a novel NOSred domain in the N-terminal of the protein containing a 2Fe2S ferredoxin subdomain. *Streptomyces turgidiscabies* also has a partially conserved zinc binding motif (Cys-X4-His). Most prokaryotes produce tetrahydrofolate (THF) instead of the cofactor BH_4. ? indicates that the co-factor that replaces BH_4 in Ostreococcus is unknown.

DIVERSITY OF NOS STRUCTURE IN PROKARYOTES

Most bacterial NOSs have been described in Gram-positive bacteria and consist of the NOSoxy domain lacking of the C-terminal NOSred domain (**Figure 1**). NOSoxy from bacteria are similar to animal NOSoxy (Crane et al., 2010). Several studies indicate that bacterial NOS use redundant cellular reductases as electron donors for the catalytic activity (Gusarov et al., 2008). As in Ostreococcus, most bacteria do not synthesize the cofactor BH_4 and thereby, they probably use tetrahydrofolate (THF) required for NOS activity (Adak et al., 2002a,b). NOSs from bacteria do not contain the CaM binding motif (Crane et al., 2010). Actually, CaM has not been identified in bacteria suggesting that CaM domain is indeed exclusive for eukaryotic NOS. Most bacterial NOS lacks tetrahedral zinc center, with the exception of NOS from *Streptomyces turgidiscabies*, where one of the two Cys is conserved and the other is replaced by His (Kers et al., 2004). Bacterial NOSs also work as homodimers. Excitingly, the discovery of NOS from the Gram-negative bacteria *Sorangium cellulosum* (scNOS) resulted in a different and novel NOS structure. ScNOS is the only characterized bacterial NOS with a covalently attached reductase domain (NOSred). This reductase module has a 2Fe2S ferredoxin domain, a FAD- binding motif and a NAD-binding motif. Interestingly, scNOS has an inverted structure: the NOSred domain is located at the N-terminal and NOSoxy at the C-terminal (Agapie et al., 2009). A similar NOS structure was found in the cyanobacteria *Microcoleus vaginatus* and *Crinalium epipsammum* (accession number ZP_08493682 and YP_007142230 respectively), although it still remains to be confirmed the NOS activity of these proteins.

The lack of apparent NOS in the plants which are most commonly worked on has led to suggest that either plants have lost this gene in the course of evolution or the gene has strongly diverged to a yet unknown new type of NOS. Since several evidences support an arginine-dependent NO production in higher plants reminiscent of a NOS activity (Cueto et al., 1996; Caro and Puntarulo, 1999; Simontacchi et al., 2004; Corpas et al., 2006, 2009; Flores et al., 2008), more efforts should be made to identify this elusive NOS form. It is noteworthy that among the NOS structures described so far, few differences were detected in the NOSoxy domain indicating that might be the core of the enzyme. Therefore the search of new NOS isoforms that differ in the NOSoxy domain would probably be the key to unravel the molecular evolution of this domain and the presence of this protein in the plant kingdom.

Overall, the unexpected diversity of the NOS structures that are currently reported in the literature should allow us to keep optimistic for identifying the NOS gene/s or protein/s complex responsible of NO generation from L-arginine in higher plants.

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Striking a balance: does nitrate uptake and metabolism regulate both NO generation and scavenging?

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Nitric oxide (NO) influences many aspects of plant development and responses to stress.The concentration of NO can play an important role in influencing its action (for example, in stomatal regulation; Wilson et al., 2009) so that the mechanisms through which NO content is modulated must be an important facet of NO research. Whilst NO generation mechanisms are clearly important, NO removal is of equal relevance, especially as plants will be continually exposed to NOx ($NO + NO_2$) gases derived from soil microbial activity (Mur et al., 2013). Establishing and regulating a poise between NO generation, NO fumigation from external sources and NO scavenging, which also needs to be flexible enough to change in response to a variety of physiological cues, is an under-considered aspect of plant NO biology.

HOW ARE NO GENERATION MECHANISMS INTEGRATED AND REGULATED?

Initially, many sought to find an equivalent to the mammalian Nitric Oxide Synthase (NOS) enzymes in plants. NOS is a cytochrome P450-like enzyme which oxidizes arginine to citrulline to generate NO (Gorren and Mayer, 2007). However, the existence of this enzyme in higher plants is still debatable (Frohlich and Durner, 2011) and is mainly based on pharmacological evidence and assays for NOS-like enzyme activity reviewed by Frohlich and Durner (2011) and Mur et al. (2013). In this context, it is also relevant that arginase mutants in Arabidopsis also displayed increased NO levels (Flores et al., 2008). However, NOS-activity has not been linked to a given gene. Resolution of this conundrum may derive from the observation that polyamine leads to NO production from Arabidopsis roots (Tun et al., 2006). As L-arginine is a precursor to polyamine biosynthesis, any perturbance of L-arginine metabolism would affect any polyamine-mediated NO generation mechanism and would explain the effects of NOS-inhibitors without needing NOS. Such a mechanism would be easily linked to the most well-characterized plant NO mechanism which is based on nitrate reductase (NR). NR acts by reducing nitrite to NO with NAD(P)H acting as an electron donor. NR-generated NO has been shown to regulate floral development, root formation, stomatal opening, and responses to biotic and abiotic stresses [reviewed in Mur et al. (2013)]. NR has high affinity for nitrate but switches to its lower affinity substrate nitrite to produce NO (Planchet et al., 2005). Therefore, NR requires high nitrite concentrations to produce NO; and a low pH is also required. Considering both NR and NOS-like NO generation mechanisms together it is possible to suggest some regulatory nodes. Thus, NO generation can be regulated at the level of NO_3^- uptake via nitrate channels, post-translational modification of NR activity (Mur et al., 2013), influencing NO_2 availability, pH and the expression and/or activity of any of the amino acid and polyamine biosynthetic enzymes. These potential regulatory mechanisms need to be systematically assessed.

Interestingly, NO_3^- also plays a central role in anoxic/hypoxic NO generation. Under hypoxia, the resulting energy crisis leads to a decrease in pH which inhibits plasidal NiR, leading to NO_3^- accumulation and NO production (Ferrari and Varner, 1971). NADH-dependent NO_3^- reduction occurs at the mitochondrial inner membrane, via cytochrome c oxidase and/or reductase and possibly by alternative oxidase (AOX) leading to the production of NO and ATP (Stoimenova et al., 2007). NO production via this mechanism occurs below 1% oxygen with a Ki value of 0.05% (0.6 μM) (Gupta and Igamberdiev, 2011). Again NO_3^- and now also NADH are limiting factors and represent possible important regulatory steps and could be the mechanism through which nitrite is transported to mitochondria which is currently not known.

Regulating the availability of NO_3^- also seems to be important in other less well-characterized NO generation mechanisms. NO may be generated in the peroxisome by a xanthine oxidoreductase (XOR) which can reduce NO_3^- to NO (Del Rio et al., 2004). NO is also generated by a plasma membrane nitrite:NO reductase (NiNOR) where NO_3^- is supplied by an apoplasmic, plasma membrane-bound NR.

BALANCING THE EQUATION: MECHANISMS OF NO REMOVAL

In planta NO content must represent the net of rates of production minus scavenging. These scavenging mechanisms must be highly efficient in order to maintain appropriate NO poise in crop species where the extensive use of nitrogen-fertilizers can result in external fumigation at rates that may be in excess of 20 nmol m^{-2} h^{-1} (Voldner et al., 1986; Benkovitz et al., 1996). Various means to reduce NO content have recently emerged; perhaps the most important being nonsymbiotic forms of hemoglobin (Hb). Oxygenated ferrous (Fe^{2+}) Hb converts NO to NO_3^- and becomes MetHb (ferric, Fe^{3+}) (metamoglobin) form which is then reduced to oxygenated ferrous (Fe^{2+}) by metamoglobin reductase (MetHb) (Hill, 2012). NO oxidation by Hb plays an important role in NO accumulation during stress

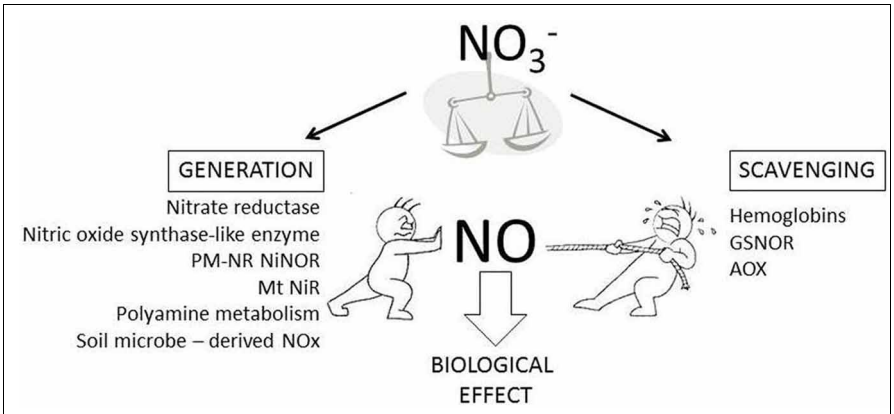

FIGURE 1 | In planta sources of NO generation and scavenging. *In planta* NO content reflects the net effect of NO generation (shown as "push" in the Figure) and scavenging (shown as "pull" in the Figure) mechanisms. NO generation can involve the listed pathway (PM-NR NiNOR, plasma membrane associated nitrate reductase coupled to nitrite reductase; Mt NiR, mitochondrial nitrite reductase). The likely role of NO_3^- in regulating *in planta* NO content is highlighted.

(Hebelstrup et al., 2012; Mur et al., 2012) thus the regulation of Hb expression is vitally important to understanding how NO poise is established (Mur et al., 2013). It is highly relevant that NO_3^- induces Hb (Wang et al., 2000) again showing how NO_3^- regulates NO content, on this occasion by influencing NO scavenging.

Other enzymes through which NO effects are modulated include S-Nitrosoglutathione Reductase (GSNOR). NO reacts with glutathione GSH and forms S-nitrosoglutathione (GSNO), which represents a significant reservoir for NO (Sakamoto et al., 2002). GSNO levels are controlled by GSNOR with converts GSNO into glutathione and sulphinamide using NADH as electron donor. Thus, GSNOR represents a means through which NO signaling may be suppressed as has been demonstrated using GSNOR mutants (Feechan et al., 2005). Additionally, under aerobic conditions mitochondria are highly efficient NO scavengers (87% of supplied NO −180 pmol) (Gupta et al., 2005). Mechanistically, this has been linked to AOX via leaking electron flow from the electron transport chain to terminal electron acceptor oxygen or nitrite in the cytochrome pathway (Cvetkovska and Vanlerberghe, 2012).

This opinion piece seeks to highlight some key questions regarding how *in planta* NO content is regulated (**Figure 1**). In developing these questions we have highlighted the role of NO_3^-. We suggest that understanding the regulation of NO_3^- uptake, assimilation and processing into a myriad of biosynthetic pathways will be central to understanding how *in planta* NO content is established.

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Functions of *S*-nitrosylation in plant hormone networks

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In plants, a wide frame of physiological processes are regulated in liaison by both, nitric oxide (NO) and hormones. Such overlapping roles raise the question of how the cross-talk between NO and hormones trigger common physiological responses. In general, NO has been largely accepted as a signaling molecule that works in different processes. Among the most relevant ways NO and the NO-derived reactive species can accomplish their biological functions it is worthy to mention post-translational protein modifications. In the last years, *S*-nitrosylation has been the most studied NO-dependent regulatory mechanism. Briefly, *S*-nitrosylation is a redox-based mechanism for cysteine residue modification and is being recognized as a ubiquitous regulatory reaction comparable to phosphorylation. Therefore, it is emerging as a crucial mechanism for the transduction of NO bioactivity in plants and animals. In this mini-review, we provide an overview on *S*-nitrosylation of target proteins related to hormone networks in plants.

Keywords: nitric oxide, phytohormones, redox mechanism, signaling, *S*-nitrosylation

INTRODUCTION

Nitric oxide (NO) is a free-radical product of cell metabolism, being nitrate reductase the best characterized enzymatic pathway for NO production in plants. However, other reductive and oxidative routes have been also described (Lamattina and Polacco, 2007). It functions as a ubiquitous signal involved in diverse physiological processes and it is frequently implicated in multiple cell signaling events under the control of phytohormones including growth, development, and stress responses. Nevertheless, in most cases the molecular mechanisms underlying NO action in the plant cell are still undeciphered. The overlapping roles between plant hormones and NO raise the question of how both molecules may act in coordination. In general, regulatory effects of NO are mediated through protein modifications, including tyrosine nitration, metal nitrosylation, and *S*-nitrosylation of cysteines. Thus, the identification of NO primary targets has provided new opportunities to link NO reactivity and biological processes. In this review, we highlight the progress brought by the identification of *S*-nitrosylated target proteins related to stress and growth-promoting plant hormones. Our focus is the broad role of this post-translational modification that allows NO to modulate plant hormone homeostasis as well as signaling pathways. However, the participation of NO beyond its action through *S*-nitrosylation in hormone-regulated processes is out of the scope of this work and it is widespreadly covered in recent reviews by Simontacchi et al. (2013) and Astier and Lindermayr (2012).

S-NITROSYLATION AS AN EMERGING POST-TRANSLATIONAL MODIFICATION OF PLANT PROTEINS

S-nitrosylation is the reversible binding of a NO moiety to a reactive cysteine residue of a target protein to form an *S*-nitrosothiol (SNO; Stamler et al., 2001). It is recognized as a reversible and ubiquitous regulatory reaction. Thus, like in animals, this redox-based post-translational mechanism is also crucial for the transduction of NO bioactivity in many plant cellular responses (Hess et al., 2005). At first, protein *S*-nitrosylation was thought to be controlled mainly through the regulation of NO biosynthesis. However, in mammals it has been postulated as a short-range NO post-translational mechanism limited to proximity of NO sources (Martinez-Ruiz et al., 2013). In addition to the enzymatic NO-producing enzymes, it is important to consider that both, favorable environment to *S*-nitrosylating agent formation as well as transnitrosylating reactions could promote the expansion of the *S*-nitrosylation range of action (Martinez-Ruiz et al., 2013). The SNO turnover could also provide an alternative mechanism to control protein *S*-nitrosylation in the cell. Given the labile nature of this post-translational modification, it was conceived initially as a spontaneous and non-regulated process. However, different denitrosylase enzymes have been described, which directly mediate denitrosylation or govern the cellular equilibrium between protein and low-molecular weight SNOs. Two main enzymatic systems have emerged as physiologically relevant denitrosylases: the glutathione/*S*-nitrosoglutathione reductase (GSH/GSNOR) and the thioredoxin/thioredoxin reductase (Trx/TrxR; Benhar et al., 2009). *S*-nitrosylation of the major intracellular antioxidant tripeptide GSH forms *S*-nitrosoglutathione (GSNO) that functions as a mobile reservoir of NO. Consequently, the enzyme GSNOR or GSNOR1 in *Arabidopsis* does not display a direct denitrosylase activity but controls intracellular levels of both, GSNO and SNO affecting the global level of *S*-nitrosylation (Feechan et al., 2005; Malik et al., 2011). On the other side, the mechanism described in animals for Trx denitrosylation involves direct interaction

with SNO-proteins by formation of an intermolecular disulphide intermediate in which Trx is covalently linked to the substrate protein through a disulphide bridge, or transnitrosylation in which Trx is transiently *S*-nitrosylated (Benhar et al., 2009). Trx have been also described in the denitrosylation process taking part in hormonal signaling in plants (Tada et al., 2008). Therefore, it appears that the balance between *S*-nitrosylation/denitrosylation is critical for the precise transduction of NO signal.

S-glutathionylation is the post-translational modification of protein cysteine residues by the addition of GSH (Martinez-Ruiz and Lamas, 2007). The integrative interplay between protein *S*-glutathionylation and *S*-nitrosylation could be recognized as another crucial network for post-translational modification of certain proteins. Although the *S*-glutathionylation of proteins has been generally described more than 20 years ago, the identification of protein targets for this modification remains rather unexplored. Interestingly, for some mammal proteins involved in clinical disorders such as cardiovascular disease and diabetes among others, *S*-nitrosylation has been described as an intermediate for more stable modifications like *S*-glutathionylation (Martinez-Ruiz and Lamas, 2007). In summary, *S*-nitrosylation is crucial for NO signal transduction pathway but it should also be noted that other related-*S*-nitrosylation regulators can converge in NO-mediated protein functionality in plants.

S-NITROSYLATION OF TARGET PROTEINS LINKED TO STRESS PHYTOHORMONES

Salicylic acid (SA) and ethylene (ET) are key signaling molecules for plants in the resistance to biotic stress (Fujita et al., 2006; Loake and Grant, 2007). NO has an essential role in restriction of pathogen attack by induction of the defense response and programed host cell death (reviewed by Mur et al., 2013). Thus, NO bioactivity may exert a role on SA and ET hormone signaling pathways.

In *Arabidopsis*, one of the first comprehensive proteomic studies allowed the identification of more than 100 *S*-nitrosylated proteins (Lindermayr et al., 2005). Interestingly, one of the identified *S*-nitrosylated proteins corresponded to a methionine adenosyltransferase (MAT) which catalyzes the synthesis of *S*-adenosylmethionine (SAM), a substrate for ET biosynthesis. Later on, Lindermayr et al. (2006) provided the first detailed molecular characterization of an *S*-nitrosylated target protein in plants. This study describes the *S*-nitrosylation of Cys-114 residue of the MAT1 isoform and the consequently inhibition of its activity. The enzymes *S*-adenosylhomocysteinase and cobalamin-independent methionine synthase are also part of the methylmethionine cycle and both enzymes have been found to be *S*-nitrosylated in proteomic analysis in *Arabidopsis* and *Kalanchoe pinnata* plants (Lindermayr et al., 2005; Abat et al., 2008). Activation/inactivation of these enzymes controls the SAM pool impacting in ET biosynthesis. All these evidences point out a multi-step control of ET biosynthesis by *S*-nitrosylation and opened the possibility to elucidate new mechanisms of NO and ET cross-talk (**Figure 1A**).

Salicylic acid is synthesized by plants in response to pathogen infection and is essential to the establishment of resistance mechanisms, including host cell death and systemic acquired resistance. Mutations in *AtGSNOR1* showed a pivotal role in the GSNO turnover, influencing cellular SNO levels under both, basal conditions and attempted microbial attack (Feechan et al., 2005). Interestingly, in the absence of AtGSNOR1 both SA biosynthesis and signaling are affected, suggesting that *S*-nitrosylation may control at least, two nodes of the SA-signaling network. GSNOR1 regulates the *S*-nitrosylation extent of non-expresser of pathogenesis-related gene1 (NPR1) and SA binding protein 3 (SABP3; Tada et al., 2008; Wang et al., 2009). *S*-nitrosylation of SABP3 is triggered during bacterial infection and suppresses SA binding capacity and carbonic anhydrase (CA) activity (Wang et al., 2009). Since, CA activity is required for the establishment of plant disease resistance, its inhibition by *S*-nitrosylation during late infection stages could contribute to a negative feedback loop which could be crucial for the proper modulation of SA-dependent plant defense mechanism (**Figure 1B**).

S-nitrosylation also exerts a key redox control of systemic acquired resistance in plants through targeting NPR1/TGA1 system. The SA NPR1-dependent signaling mechanism is mediated by redox changes that lead to reduction of NPR1 cysteines. This event switches NPR1 from cytosolic, disulfide-bound oligomers, to active monomers that are subsequently translocated into the nucleus and interacts with the TGA class of basic leucine zipper transcription factors. The result is an enhanced binding activity of TGA1 to the promoter region of pathogenesis-related (PR) genes, stimulating SA-dependent immune defense (Vlot et al., 2009). Upon pathogen attack, SA induces Trx which facilitates NPR1 monomerization, nuclear translocation, and activation of PR genes (Tada et al., 2008). Additionally, Tada et al. (2008) demonstrated that NPR1 is an *S*-nitrosylated protein. Notably, TGA1 is regulated by *S*-nitrosylation and *S*-glutathionylation improving TGA1 binding activity to PR1 promoter region (Lindermayr et al., 2010). However, it has not been demonstrated which type of modification, *S*-nitrosylation, *S*-glutathionylation, and/or both, is responsible for such protein–DNA binding activity (**Figure 1B**).

Concluding, plant immunity is regulated by a precise redox balance between the opposing actions of distinct redox-signals that catalyze NPR1 oligomer–monomer switch and NPR1/TGA1 interaction through transient redox fluctuations that includes *S*-nitrosylation and *S*-glutathionylation. Moreover, in the cytosol NPR1 also contributes to the suppression of jasmonic acid (JA)-dependent responses (Spoel et al., 2003), evidencing *S*-nitrosylation as a mediator of the integrative hormonal regulation network for guarantee immunity in plants.

Meanwhile, abscisic acid (ABA) is the major player mediating adaptation of plants to drought stress. ABA induces stomatal closure and inhibits stomatal opening by facilitating osmotic solute loss to reduce guard cell turgor. These events take place through a complex signaling network that involves multiple components including Ca^{2+}, K^+, IP_3, MAPK, and H_2O_2 (Fan et al., 2004). NO enhances plant tolerance to drought and it contributes to stomatal closure evoked by ABA. Mechanistically, NO regulates inward-rectifying K^+ channels through its action on Ca^{2+} release from intercellular stores. Alternative pathways have been also indicated for NO action on the outward-rectifying K^+ channels, which are Ca^{2+} insensitive. It is probable that NO directly modifies the K^+

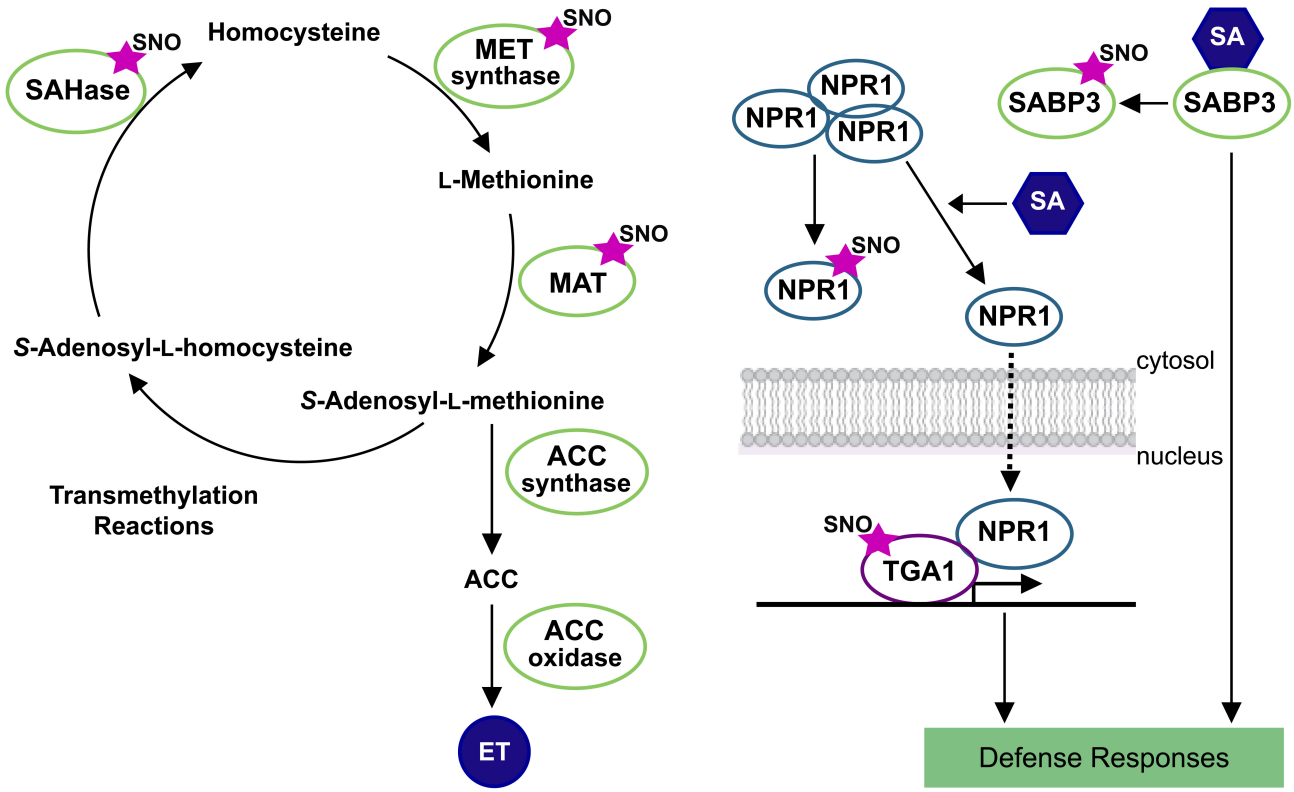

FIGURE 1 | *S*-nitrosylation of target proteins in ethylene biosynthesis and salicylic acid network. The figure shows a schematic representation of methylmethionine cycle in the ethylene (ET) synthesis **(A)** and salicylic acid (SA) signaling networks **(B)**. Protein *S*-nitrosothiols are represented by an SNO mark. References to physiological processes regulated by hormones, and subcellular localizations in the cell are also indicated. SAHase, adenosylhomocysteinase; MET synthase, cobalamin-independent methionine synthase; MAT, methionine adenosyltransferase; ACC, aminocyclopropane-1-carboxylic acid; SABP3, salicylic acid binding protein 3; NPR1, non-expresser of pathogenesis-related gene1 protein; TGA1, transcription factor TGA1.

channel at the guard cell plasma membrane or a closely associated regulatory protein through *S*-nitrosylation (Sokolovski and Blatt, 2004). However, the physiological significance of this regulation remains unexplored.

TARGETS FOR PROTEIN *S*-NITROSYLATION IN SIGNALING PATHWAYS OF GROWTH-PROMOTING PHYTOHORMONES AUXINS AND CYTOKININS

Auxins and cytokinins (CKs) are critical regulators of cell division, expansion, and differentiation. Relatively recent breakthroughs were found by comparing functions of NO and the well-known growth-promoting hormones (reviewed by Mur et al., 2013). There are several examples of NO and auxin overlapping effects during shoot and root organogenesis such as, NO mediation of auxin-induced adventitious and lateral roots (Pagnussat et al., 2002; Correa-Aragunde et al., 2004), root hair formation (Lombardo et al., 2006), and adventitious root formation (Pagnussat et al., 2003). NO stimulates the activation of cell division and embryogenic cell formation in leaf protoplast in the presence of auxin (Otvos et al., 2005). Copper-induced morphological responses are also mediated by auxin and NO in *Arabidopsis* seedlings (Peto et al., 2011). All these previous evidences led to investigate the possible interplay between these two signal molecules. Briefly, in the case of auxin, its perception is mediated by the F-box protein TIR1 (transport inhibitor response1) and the related proteins, AUXIN SIGNALING F-BOX proteins (AFBs; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Auxin binding stabilizes the interaction between TIR1/AFBs and the transcriptional repressor proteins, auxin/indole-3-acetic acid (Aux/IAA) causing a rapid proteasomal degradation of them (Gray et al., 2001). Then, Aux/IAA degradation results in the activation of transcriptional responses with the concomitant impact in plant growth and development (Tan et al., 2007). In an attempt to study the possible mechanism by which NO might regulate auxin signaling, *S*-nitrosylation of auxin receptor was analyzed. *S*-nitrosylation of TIR1 was demonstrated by Terrile et al. (2012). This redox-based modification enhances the efficiency by which TIR1 interacts with Aux/IAAs facilitating their degradation and modulating auxin signaling during root growth in *Arabidopsis* seedlings (**Figure 2**). Particularly, Cys-140 is a critical residue for TIR1–Aux/IAA interaction and TIR1 function. *S*-nitrosylation of TIR1 represents an efficient mechanism by which NO might enhance sensitivity and/or ligand selectivity. Furthermore, NO modulation of auxin signaling is more complex

since a combinatorial TIR1/AFB–Aux/IAA co-receptor system could be assembled, contributing to the versatility of auxin response (Calderon Villalobos et al., 2012). However, cellular effectors of denitrosylation remain to be explored. Recently, Correa-Aragunde et al. (2013) described a new convergence where auxins are thought to influence *S*-nitrosylation/denitrosylation balance in *Arabidopsis* roots. The antioxidant enzyme, APX1 is an *S*-nitrosylation target and auxin induces denitrosylation and partial inhibition of its activity (Correa-Aragunde et al., 2013). These authors postulated that an auxin-regulated balance of APX1 *S*-nitrosylation/denitrosylation state contributes to a fine-tuned control of reactive oxygen species (ROS) that finally impacts on root architecture and development. Recent studies have pointed out the correlation between ROS and auxin homeostasis in signal transduction during plant development and stress response (Tognetti et al., 2012). In this direction, Bashandy et al. (2010) also highlighted the intercellular redox status as a critical parameter determining plant development through modulation of auxin signaling, transport, and homeostasis. Although our knowledge about auxin and NO is currently being born, most probably *S*-nitrosylation/denitrosylation is of great impact throughout to interlink these two molecules along plant lifecycle.

Plant hormones CKs are well known for their ability to promote cell division and they are associated with growth and development, including lateral root formation and nodulation in legumes (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007), circadian rhythms (Salome et al., 2006), and shoot and root development (Werner and Schmulling, 2009). Recently, NO-mediated CK functions have been associated to cell proliferation and meristem maintenance in *Arabidopsis* (Shen et al., 2013). CKs are perceived and mediated by a multi-step two-component circuit through a histidine and aspartate phosphorelay (Muller and Sheen, 2007). CKs regulate their signals through a variety of mechanisms, such as modulating transcription, controlling phosphorelay and regulating protein localization and stability (To and Kieber, 2008). In a recent report, Feng et al. (2013) demonstrated that NO represses CK signaling by inhibiting the phosphorelay activity through *S*-nitrosylation. Interestingly, the authors showed that NO-overproducing mutants, *nox-1* (*NO overproducer1*) and *gsnor1–3* do not respond to CK-induced shoot regeneration in *Arabidopsis* explants. Moreover, *gsnor1–3* has a substantial reduction on the expression of the primary response regulator genes (ARRs) for CK signaling. Centrally, by the use of an *in vivo* biotin-switch assay, it was demonstrated that the histidine phosphotransfer protein AHP1 is *in planta* *S*-nitrosylated under normal growth conditions. Cys-115 was proposed as an *S*-nitrosylated residue. Comprehensively, AHP1 *S*-nitrosylation compromises CK action revealing again, a mechanism through which CK signaling components perceive and integrate a redox signal in the regulation of plant growth and development (**Figure 2**). Although several lines of evidence support the involvement of NO in CK signaling (Carimi et al., 2005; Tun et al., 2008), other works claim an opposite effect of NO in CK action (Werner et al., 2003; Riefler et al., 2006; Xiao-Ping and Xi-Gui, 2006). Much more recently, a direct interaction between NO and CK has been also described (Liu et al., 2013). In summary, NO roles could be of the most varied because in addition to its own action it meets specific cellular functions according to the target molecules amending within the routes of hormonal regulation in plant cells.

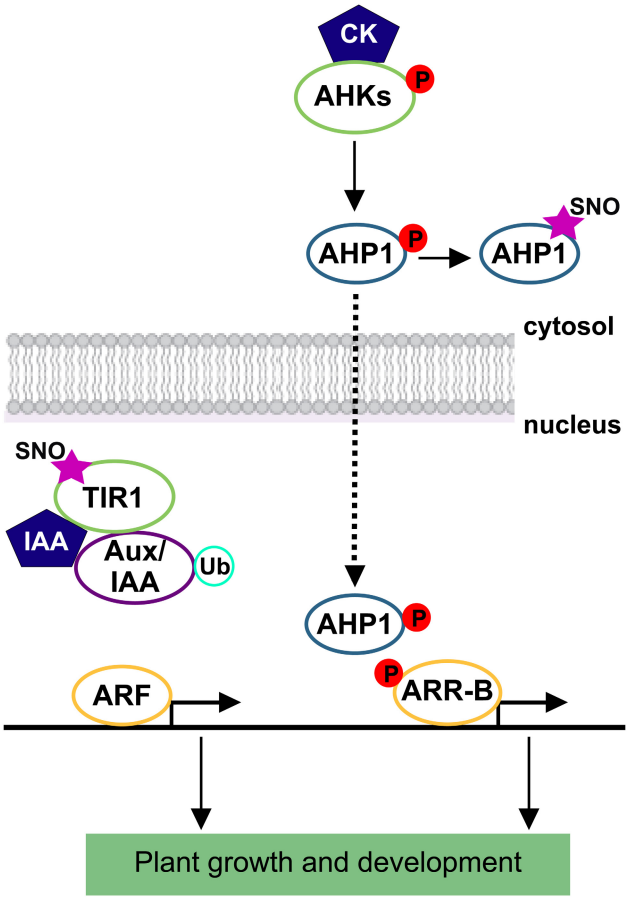

FIGURE 2 | Auxin and cytokinin signaling pathways are under protein *S*-nitrosylation influence. Overview of indole-acetic acid (IAA) and cytokinin (CK) signaling pathways. *S*-nitrosothiols are represented by an SNO mark. Protein phosphorylation is represented by a P letter. References to physiological processes regulated by hormones, and subcellular localizations are also indicated. TIR1, transport inhibitor response 1 protein; Aux/IAA, auxin/indole-3-acetic acid protein; ARF, auxin response factor; Ub, ubiquitin; AHKs, hybrid histidine protein kinases; AHP1, histidine phosphotransfer protein 1; ARR-B, primary response regulator type B.

CONCLUDING REMARKS AND PERSPECTIVES

NO is a fascinating molecule with remarkable feats and properties to modulate signaling pathways in biological systems. The bioactivity of NO is high enough for it to occur in a wide variety of biochemical circumstances. *S*-nitrosylation/denitrosylation is currently accepted as critical redox-mediated regulation processes in plant cells. Certainly, *S*-nitrosylation could be a possible mechanism by which NO impacts on plant hormonal regulation by modulating hormone biosynthesis, perception, transport, and/or degradation. Clearly, multiple layers of interactions may be involved in the plant hormones and NO cross-talks, depending on complex biological and biochemical scenarios in cells. However,

nowadays fragmented studies on its *in vivo* function hamper our thorough understanding on hormone–NO cross-talking. Probably, high-throughput genetic and protein-based approaches in combination with a deeper understanding on the basic structure/function relationships of NO generating systems will shed light on this scientific riddle.

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Effect of nitric oxide on gene transcription – *S*-nitrosylation of nuclear proteins

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Nitric oxide (NO) plays an important role in many different physiological processes in plants. It mainly acts by post-translationally modifying proteins. Modification of cysteine residues termed as *S*-nitrosylation is believed to be the most important mechanism for transduction of bioactivity of NO. The first proteins found to be nitrosylated were mainly of cytoplasmic origin or isolated from mitochondria and peroxisomes. Interestingly, it was shown that redox-sensitive transcription factors are also nitrosylated and that NO influences the redox-dependent nuclear transport of some proteins. This implies that NO plays a role in regulating transcription and/or general nuclear metabolism which is a fascinating new aspect of NO signaling in plants. In this review, we will discuss the impact of *S*-nitrosylation on nuclear plant proteins with a focus on transcriptional regulation, describe the function of this modification and draw also comparisons to the animal system in which *S*-nitrosylation of nuclear proteins is a well characterized concept.

Keywords: protein *S*-nitrosylation, nitric oxide, post-translational modification, nuclear proteins, redox-modification

INTRODUCTION

Nitric oxide (NO) is a small, highly reactive gaseous radical. Although it is cytotoxic in high concentrations, NO plays a key role as a biological messenger in all kingdoms. In plants, it is implicated in various physiological processes like flowering, stomatal closure, germination, root development, gravitropism, and responses to abiotic and biotic stresses (Delledonne et al., 1998; Durner et al., 1998; Garcia-Mata and Lamattina, 2002; Pagnussat et al., 2002; He et al., 2004; Hu et al., 2005; Lombardo et al., 2006; De Michele et al., 2009; Sirova et al., 2011; Tanou et al., 2012).

Due to its instable nature, NO has a very rich chemistry. Besides direct dative binding to metal ions NO can further react with superoxide and molecular oxygen, resulting in the formation of peroxynitrite and dinitrogen trioxide N_2O_3 (or higher oxides like NO_2), respectively. Moreover, adding or removing one electron from the antibonding highest occupied molecular orbital by reducing or oxidizing chemicals yields nitroxyl anion (NO^-) and nitrosonium cation (NO^+). Collectively, these species are referred to as reactive nitrogen species (RNS) each having distinct chemical properties leading to numerous reactions with biological molecules like lipids, carbohydrates, nucleic acids, and proteins. Although most of these reactions were assumed to be indicative for nitrosative stress in the past, it has become clear that some of these RNS also function as important redox-signaling molecules in the cell by binding covalently to target proteins (Suzuki et al., 2012; Yun et al., 2012). This as redox-signaling termed mechanism should not be considered as a discrete set of signaling cascades. Rather, the cell should be seen as set of compartments each having distinct redox-sensitive proteins as well as redox buffering capacities. Changes in the redox potential of these compartments could then influence other signaling pathways by modifying redox-sensitive proteins (Foyer and Noctor, 2013).

There are three important NO-dependent modifications: metal nitrosylation, tyrosine nitration, and cysteine *S*-nitrosylation.

In a direct reaction termed metal nitrosylation, NO (Lewis base) binds to the transition metal (Lewis acid) of metalloproteins yielding a metal–nitrosyl complex. One example from mammals is the binding of NO to the heme center of soluble guanylate cyclase which activates this enzyme by inducing conformational changes and this in turn leads to the production of cyclic GMP (Russwurm and Koesling, 2004).

Reactive nitrogen species can modify the activity of proteins by covalently binding to tyrosine and cysteine residues. Tyrosine nitration refers to the addition of a nitro group to susceptible tyrosine residues in ortho position to the hydroxyl group thus leading to 3-nitrotyrosine. The main nitrating species is peroxynitrite which is produced in a diffusion controlled reaction between NO and superoxide (Ferrer-Sueta and Radi, 2009). Tyrosine nitration was originally considered to be indicative for oxidative and nitrosative stress but evidence accumulates that this modification also has a signaling function in plant cells (Cecconi et al., 2009; Gaupels et al., 2011).

S-nitrosylation of protein cysteine residues is believed to be the most important mechanism for transduction of bioactivity of NO in plants. The formation of nitrosothiols is still debated. The direct reaction of thiol groups with NO is too slow to occur *in vivo*, instead it is assumed that N_2O_3 is the main nitrosylating species in aerobic conditions although the formation of dinitrogen trioxide is controversially discussed (Folkes and Wardman, 2004; Ridnour et al., 2004). Other RNS described to mediate *S*-nitrosothiol formation are nitrosonium and nitroxyl ions (Ridnour et al., 2004). Nitroso groups can also be transferred between thiols in a process termed as transnitrosylation. Transnitrosylation occurs between proteins and between proteins and low molecular weight SNOs

(e.g., *S*-nitrosylated glutathione GSNO) in animals; in plants, however, evidence for this mechanism is lacking (Hogg, 2002; Nakamura and Lipton, 2013). Enzymatic denitrosylation is mediated by GSNO reductase (GSNOR) and thioredoxins (Trx), both proteins are crucial for maintaining SNO-homeostasis (Sakamoto et al., 2002; Feechan et al., 2005; Sengupta and Holmgren, 2013).

Initial proteomic screens for *S*-nitrosylated proteins in *A. thaliana* revealed 53 mainly cytoplasmic proteins but this number increased drastically over the last years (Lindermayr et al., 2005). Up to date several screens targeting the proteomes of different organelles like mitochondria and peroxisomes identified more than 250 candidate proteins to be *S*-nitrosylated involved in a wide range of physiological processes ranging from stress response to metabolism (Kovacs and Lindermayr, 2013; Lounifi et al., 2013). Interestingly, microarray analysis and amplified fragment-length polymorphism (AFLP) transcript profiling of plants treated with gaseous NO and sodium nitroprusside, respectively, showed that NO leads to changes in the transcriptome of *Arabidopsis* (Huang et al., 2002; Polverari et al., 2003). Promoter analysis of the genes co-expressed after NO treatment revealed the accumulation of certain transcription factor binding sites, like octopine synthase gene (ocs) elements and WRKY-sites (Palmieri et al., 2008). This raised the question whether NO affects transcription directly by nitrosylating transcription factors or other transcriptional regulators. In some bacteria, for instance, redox-sensitive cysteine residues of the transcriptional activator OxyR can undergo redox-dependent post-translational modifications like oxidation to sulfinic acid, *S*-glutathionylation, or *S*-nitrosylation. Each of these modifications affects binding affinity and specificity of OxyR to DNA thus resulting in distinct transcriptional responses (Marshall et al., 2000). Besides regulation of DNA-binding, *S*-nitrosylation of nuclear proteins could also affect their subcellular localization or regulate the association with binding partners thereby modulating transcription and/or general nuclear metabolism. In animals, for instance, *S*-nitrosylation of the nuclear export receptor CRM1 (karyopherin chromosomal region maintenance 1) leads to a decrease in the export rate and a subsequent nuclear accumulation of its target protein Nrf2, an antioxidant transcription factor (Wang et al., 2009). The possible modes of action of NO on gene transcription are shown in **Figure 1**.

In this review, we will summarize the current knowledge about *S*-nitrosylated nuclear plant proteins. What is the impact and function of this post-translational modification? Comparisons to the animal system will be drawn in which much more is known about the effect of *S*-nitrosylation on transcription.

S-NITROSYLATED NUCLEAR PROTEINS

GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE AND CYTOSOLIC ALDOLASE

It is well-known that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) not only plays an important role in glycolysis but also participates in nuclear events like regulation of gene transcription,

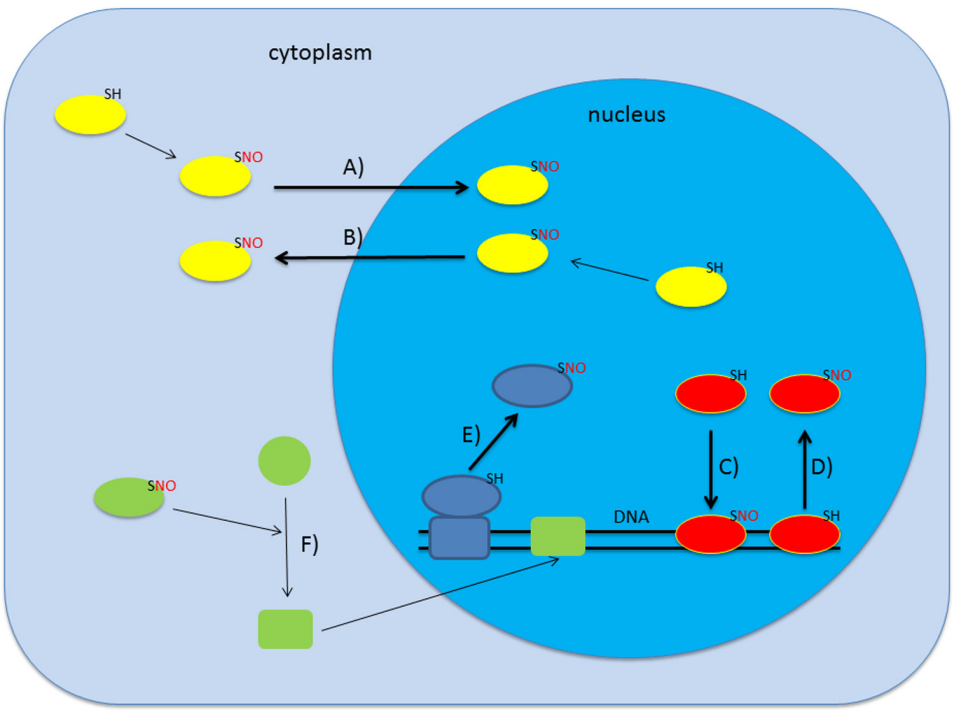

FIGURE 1 | *S*-nitrosylation can affect gene transcription in several ways. Upon *S*-nitrosylation proteins can change their subcellular localization which may lead to either import in **(A)** or export out **(B)** of the nucleus (Qu et al., 2007; Malik et al., 2010). Alternatively, *S*-nitrosylation is also described to alter DNA-binding activity of certain proteins (**C,D**; Serpa et al., 2007; Lindermayr et al., 2010; Sha and Marshall, 2012). Additionally, SNO-formation can lead to association/dissociation of macromolecular complexes which may result in dissociation from chromatin (**E**; Nott et al., 2008). Various combinations including indirect regulation are also conceivable **(F)**.

RNA transport and DNA replication. In animal cells, the link between NO signaling and nuclear action of GAPDH is well established. GAPDH lacks a nuclear localization signal and the homotetramer is too large (150 kDa) to pass passively through nuclear pores. Upon stress GAPDH is specifically nitrosylated at Cys150 by inducible NO-synthase (iNOS) leading to complex formation with seven in absentia homolog 1 (Siah1), an E3 ubiquitin ligase. Siah1 has a very rapid turnover in HEK293 cells but binding to GAPDH markedly increases its stability. The nuclear import signal of Siah1 enables the translocation of the GAPDH/Siah1 complex into the nucleus (Hara et al., 2005). Interestingly, it was shown that nitrosylated GAPDH can transnitrosylate nuclear proteins including deacetylating enzyme sirtuin 1 (SIRT1), histone deacetylase 2 (HDAC2), and DNA-activated protein kinase (DNA-PK) thereby affecting gene transcription (Kornberg et al., 2010). This mechanism can elegantly explain specificity of *S*-nitrosylation in the nucleus in the absence of a nuclear NO-synthase (Stamler and Hess, 2010).

In *Arabidopsis*, both GAPDH isoforms GapC1 and GapC2 were shown to be nitrosylated and glutathionylated on Cys155 and Cys159 (Holtgrefe et al., 2008). These cysteine modifications inhibit GAPDH *in vitro*, but activity could be restored upon addition of dithiothreitol (DTT) demonstrating the reversibility of these modifications. A GFP–GAPDH fusion protein was localized in both the cytosol and nucleus in *A. thaliana* protoplasts indicating partial nuclear localization of GAPDH (Holtgrefe et al., 2008). Moreover, a complex of a GAPDH isoform and NtOSAK (*Nicotiana tabacum* osmotic stress-activated protein kinase) partially localized to the nucleus in BY2 cells after salt stress. Both proteins of this complex seem to be regulated by NO: GAPDH is directly *S*-nitrosylated, whereas the regulation of NtOSAK is rather indirect, involving the NO-dependent phosphorylation of a serine residue in the activation loop of the kinase (Wawer et al., 2010). In addition, cadmium stress induced a strong nuclear accumulation of GapC1 in *Arabidopsis* root tips, which was – in sharp contrast to animal cells – not dependent on *S*-nitrosylation of the catalytic Cys-residue (Vescovi et al., 2013). Interestingly, GAPDH was found to bind to the malate dehydrogenase promoter by using electrophoretic mobility shift assays pointing toward a possible role as transcriptional activator/repressor (Holtgrefe et al., 2008). In conclusion, in contrast to animal cells, the molecular function of *S*-nitrosylation of GAPDH in plants is rather unclear, and further work is needed to decipher the role of GAPDH in NO-mediated signaling.

Aldolases catalyze the reversible condensation of D-glyceraldehyde-3-phosphate and dihydroxyacetone phosphate and are involved in glycolysis, gluconeogenesis, and the Calvin cycle. Higher plants possess different isoforms of aldolases localized to either the cytosol or plastids. It was shown that the enzymatic activity of one isoform of cytosolic aldolase from *A. thaliana* is inhibited by different redox modifications. Cys68 and Cys173 were both glutathionylated, while nitrosylation was only detected at Cys173 (van der Linde et al., 2011). Several studies support the idea that cytosolic aldolase might take over functions in the nucleus. First, this enzyme was found to be localized in the pea leaf nucleus (Anderson et al., 2005). Second, cytosolic aldolase was identified as an interaction partner of the MADS-box transcription factor NMH7 in *Medicago sativa* (Paez-Valencia et al., 2008). Third, a GFP-fusion construct partially localized to the nucleus in *A. thaliana* protoplasts (van der Linde et al., 2011). Fourth, this enzyme was shown to be associated with the NADPH-malate dehydrogenase promoter (Hameister et al., 2007). However, nothing is known about the impact of redox modifications on nuclear activity of cytosolic aldolase.

MYB TRANSCRIPTION FACTORS

In plants, MYB factors are one of the largest families of transcription factors (Stracke et al., 2001). In the genome of *A. thaliana*, approximately 9% of the estimated number of transcription factors belongs to the MYB family (Riechmann et al., 2000). MYB transcription factors are involved in the regulation of a wide range of physiological processes including metabolic pathways, cell fate and identity, developmental processes and responses to biotic and abiotic stresses (Dubos et al., 2010). They are characterized by a highly conserved DNA-binding domain (MYB DBD). The MYB DBD consists of up to four sequence repeats of about 52 amino acids, each forming three α-helices (Dubos et al., 2010). The third helix of each repeat is the "recognition helix" that makes direct contact with the major groove of DNA (Dubos et al., 2010). The repeated domains increase specificity of DNA-binding and depending on their number, MYB proteins can be divided into different classes. R2–R3 MYB factors constitute an expanded family of MYB proteins in plants that contain a N-terminal DNA-binding domain formed by two adjacent MYB repeats (R2 and R3) and an activation or repression domain usually located in the C-terminus (Dubos et al., 2010). In contrast to the highly conserved MYB domain, the other regions of R2R3-MYB proteins are highly variable which can explain the wide range of regulatory roles of members of this family in plant-specific processes (Wilkins et al., 2009). R2R3-MYB factors contain a highly conserved Cys at position 53 (Cys53) which is also present in MYB proteins from animals and fungi (Serpa et al., 2007). The presence of this surface exposed Cys-residue within the DNA-binding domain raises the question whether DNA-binding activity is regulated by oxidative modifications of this amino acid. Indeed, the DNA-binding of M2D (a fully active DNA-binding domain of AtMYB2) is inhibited by *S*-nitrosylation of Cys53 (Serpa et al., 2007). This mechanism might be conserved throughout different kingdoms as it was demonstrated that NO-donors (SNP and SNOG) severely inhibited DNA-binding of the chicken c-Myb minimal DNA-binding domain R2R3 and that Cys130 (equivalent to Cys53 in plants) is essential for this inhibitory effect (Brendeford et al., 1998).

NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 AND TGA1

In mammalian immunity, the cofactor inhibitor of kappaB (IκB), which shares structural features with plant non-expressor of pathogenesis-related genes 1 (NPR1; Cao et al., 1997; Ryals et al., 1997), functions to sequester the transcription factor nuclear factor kappaB (NF-κB) in the cytoplasm and prevents it from activating gene expression. In response to pathogen attack, IκB is rapidly phosphorylated and targeted for ubiquitin-mediated proteolysis, allowing NF-κB to localize to the nucleus and activate target genes (Hayden and Ghosh, 2004).

NF-κB itself is a redox-regulated transcription factor in mammals. Within the DNA-binding domain, Cys62 of the p50 subunit is critical for ROS-regulated DNA-binding (Matthews et al., 1992) and is modified by *S*-nitrosylation (Matthews et al., 1996).

The transcription cofactor NPR1, a key regulator of systemic acquired resistance (SAR), is essential for salicylic acid (SA)-mediated signal transduction (Rockel et al., 2002). Recently, it has been shown that NPR1 binds SA and works as a SA receptor (Wu et al., 2012). In unchallenged plants, Cys residues in NPR1 form intermolecular disulfide bonds, driving the formation of NPR1 oligomers (Mou et al., 2003). These NPR1-oligomers are retained in the cytosol. Upon pathogen challenge, the level of SA increases followed by changes in the cellular redox state, resulting in reduction of disulfide bonds in NPR1. Reduction of the NPR1 oligomers releases monomers that translocate to the nucleus where they interact with TGA transcription factors and subsequently activate the expression of pathogenesis-related (PR) genes (Kinkema et al., 2000). Moreover, NPR1 regulates the transcript accumulation of callose synthase genes during defense response (Dong et al., 2008). Interestingly, *S*-nitrosylation of C156 of NPR1 facilitates its oligomerization (Tada et al., 2008). Trx catalyze the monomerization of NPR1 and allow the translocation into the nucleus. Surprisingly, the nuclear translocation of NPR1 is also induced by GSNO (Lindermayr et al., 2010). However, the *S*-nitrosylation-mediated oligomerization is not considered to be an inhibitory effect of NPR1 signaling but rather as a step prior to monomer accumulation.

The TGACG motif binding transcription factors (TGA) belong to the group of basic leucine zipper (bZIP) proteins and the DNA-binding sites for several bZIP factors were enriched in promoter regions of NO-regulated genes (Palmieri et al., 2008). In the nucleus, NPR1 interacts with TGA that binds to *cis*-elements of the *PR1* promoter, promoting *PR1* gene expression and defense (Zhou et al., 2000; Despres, 2003). Redox-dependent interaction with NPR1 is only described for TGA1 and TGA4 which comprise group I and possess four cysteine residues. TGA2, TGA3, TGA5, TGA6, and TGA7 interact with NPR1 independently of the cellular redox status (Zhang et al., 1999; Zhou et al., 2000; Despres, 2003). The Cys residues C260 and C266 of TGA1 form a disulfide bond under oxidizing conditions precluding its interaction with NPR1. These Cys residues are conserved in TGA4, but not in the other TGA isoforms.

Redox regulation of TGA1 and NPR1 has been proposed to involve *S*-nitrosylation (Lindermayr et al., 2010). Both proteins are *S*-nitrosylated *in vitro* after *S*-nitrosoglutathione (GSNO) treatment (Tada et al., 2008; Lindermayr et al., 2010), resulting in enhanced DNA-binding activity of TGA1 toward its cognate target in the presence of NPR1 (Lindermayr et al., 2010). The GSNO-dependent modifications probably result in conformational changes of TGA1 and/or NPR1, which allow a more effective TGA1–NPR1 interaction and enhanced DNA-binding of TGA1 (Lindermayr et al., 2010). The redox status of C172/C287 of TGA1 seems to be important for its DNA-binding activity. Reducing this disulfide bridge and subsequent GSNO-dependent modification of the corresponding cysteine residues positively affect DNA-binding of this transcription factor (Lindermayr et al., 2010).

HISTONE DEACETYLASES

Acetylation of histone lysine residues is a very important epigenetic regulatory mechanism. Histone acetyltransferases (HATs) catalyze the transfer of acetyl groups from acetyl-coenzyme A on lysine residues of histone tails thereby neutralizing the positive charge of the lysine residue. This reduces the affinity of histones for negatively charged DNA resulting in a loose chromatin structure that is easily accessible for the transcriptional machinery. In contrast, histone deacetylases (HDACs) remove the acetyl group of histone tails and condense the chromatin, thereby resulting in reduced gene expression (Luo et al., 2012). Histones are not the only substrates of HATs and HDACs, acetylation and deacetylation of a wide variety of proteins is catalyzed by these enzymes (Wu et al., 2000). In animals, members of both enzyme groups are known to be regulated by *S*-nitrosylation. Here, we will focus on HDACs because so far there is nothing known about *S*-nitrosylation of HATs in plants.

Brain-derived neurotrophic factor (BDNF) and other neurotrophins play a crucial role in the development of the rat and mouse nervous system by influencing the expression of many specific genes that promote differentiation, cell survival, etc. (Nott et al., 2008). Since studies on the effect of NO on chromatin remodeling in neurons showed that NO alters the acetylation state of chromatin associated with the promoter of neurotrophin-regulated genes, one function of NO in the nucleus might be to regulate gene expression by influencing the interaction of transcription factors with chromatin (Nott et al., 2008). Nott et al. (2008) investigated whether NO affects histone acetylation by modifying HDAC activity and found that NO is a key regulator of human histone deacetylase 2 (HDAC2). It was shown that BDNF triggers NO synthesis and also a rapid and sustained *S*-nitrosylation of HDAC2 in neurons. HDAC2 contains three cysteine residues and only double mutation of Cys262 and Cys274 completely abolished its *S*-nitrosylation (Nott et al., 2008). *S*-nitrosylation of HDAC2 did not affect its deacetylase activity, in contrast, it induced its release from chromatin, which lead to an increase of histone acetylation at specific promoter regions and transcription of genes associated with neuronal development including c-fos, egr1, VGF, and nNos (Riccio et al., 2006; Nott et al., 2008). NO-dependent inhibition of HDAC2 function has also been reported in muscle cells (Colussi et al., 2008). Interestingly, *S*-nitrosylation decreases HDAC2 deacetylase activity (Colussi et al., 2008) whereas in neurons HDAC2 enzymatic activity remains unchanged (Nott et al., 2008). This divergence could be due to different *S*-nitrosylated cysteine residue(s) of HDAC2 in muscle cells and neurons (Nott and Riccio, 2009).

In mammals, class I HDACs are ubiquitously expressed and are localized predominantly in the nucleus. In contrast, class II and IV HDACs are expressed tissue-specific and they are regulated by controlling their subcellular localization (Watson and Riccio, 2009). In unstimulated cells, class II HDACs (e.g., HDAC4/5) are retained in the cytoplasm due to phosphorylation by calcium–calmodulin-dependent kinases (CaMKs) and subsequent association with the cytoplasmic chaperone 14-3-3 (McKinsey et al., 2001). Upon stimulation, dephosphorylation leads to the dissociation of the complex allowing class II HDACs to shuttle into the nucleus. Class II HDACs are indirectly regulated by NO.

S-nitrosylation of PP2A enforces its binding to HDAC4/14-3-3 leading to dephosphorylation and subsequent nuclear localization of HDAC4 (Illi et al., 2008).

In plants, three families of HDACs can be distinguished based on sequence similarity. The largest family in *Arabidopsis* consists of 12 members – characterized by a highly conserved HDAC domain – and shares homology with yeast RPD3 (reduced potassium dependency protein 3) or HDA1 (histone deacetylase 1). Sirtuins (two members in *Arabidopsis*) are homologous to yeast SIR2 (silent information regulator 2) and have a different catalytic mechanism as they need NADH as a cofactor. The HD2-like family seems to be plant-specific, no homologs have been identified in other organisms so far (Luo et al., 2012). HD2-like proteins play an important role during the hypersensitive response in tobacco: Bourque et al. (2011) showed that NtHD2a/b act as negative regulators of cryptogein induced cell death by using HDAC inhibitors, RNAi, and overexpression approaches. Alignment of *Arabidopsis* RPD3-like HDACs revealed the presence of some highly conserved cysteine residues. Interestingly, Cys262 or Cys274 of human HDAC2 (which were shown to be nitrosylated; Nott et al., 2008) are also preserved in many *Arabidopsis* HDACs (for instance Cys209 and Cys221 of HDA19), making these proteins interesting candidates for further studies. Data from our lab support the idea that histone deacetylases might also be redox regulated in plants (Floryszak-Wieczorek et al., 2012).

CONCLUSION

S-nitrosylation is emerging as one of the most important redox-dependent modifications in plants but only very few detailed studies are available about the impact of this modification on nuclear plant proteins. Important knowledge about *S*-nitrosylation in general in the nucleus is still lacking. Specifically, the presence of NO or nitrosylating species in this compartment has not been proven so far. It is also known that GSH – the main reductant of the cell – accumulates to very high concentrations in the nucleus at certain cell cycle stages, probably to protect the DNA from oxidative damage (Garcia-Gimenez et al., 2013). This raises the question how *S*-nitrosylation in the nucleus is maintained and temporally/spatially controlled. Nevertheless, evidence accumulates that *S*-nitrosylation of nuclear plant proteins (for instance transcription factors) probably participates in regulation of transcription. In animals, several transcription factors are known to be regulated by this post-translational modification: results from studies in neuronal physiology have demonstrated that NO modulates gene expression through the formation of SNO-bonds in multiple transcriptional activators (Nott and Riccio, 2009). For instance, *S*-nitrosylation mediates NO-dependent regulation of various zinc-finger-containing transcription factors, including egr-1 and NFκB. As zinc-finger motifs are very sensitive to *S*-nitrosylation this class of TFs might also be interesting to study in plants. Besides acting on transcription factors, NO also seems to be involved in epigenetic regulation of plant chromatin by modifying key remodeler enzymes like HDACs, which is a new and fascinating aspect of NO-mediated redox signaling in plants. However, important questions are remaining. Work so far has mostly been carried out *in vitro*, the *in vivo* relevance as well as the exact molecular mechanism still needs to be determined leaving much space for future investigations.

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Cross-talk of nitric oxide and reactive oxygen species in plant programed cell death

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In plants, programed cell death (PCD) is an important mechanism to regulate multiple aspects of growth and development, as well as to remove damaged or infected cells during responses to environmental stresses and pathogen attacks. Under biotic and abiotic stresses, plant cells exhibit a rapid synthesis of nitric oxide (NO) and a parallel accumulation of reactive oxygen species (ROS). Frequently, these responses trigger a PCD process leading to an intrinsic execution of plant cells. The accumulating evidence suggests that both NO and ROS play key roles in PCD. These redox active small molecules can trigger cell death either independently or synergistically. Here we summarize the recent progress on the cross-talk of NO and ROS signals in the hypersensitive response, leaf senescence, and other kinds of plant PCD caused by diverse cues.

Keywords: nitric oxide, reactive oxygen species, programed cell death, hypersensitive response, leaf senescence

NO, REACTIVE NITROGEN SPECIES, AND PROTEIN *S*-NITROSYLATION

Nitric oxide (NO) is a gaseous free radical which was first found to play a crucial role in plant and mediating defense reactions against bacterial pathogens (Noritake et al., 1996; Delledonne et al., 1998). Increasing evidence suggests that NO, as a signal mediator, plays a key role in many physiological and developmental processes, such as germination, leaf expansion, lateral root development, flowering, stomatal closure, crosstalk with plant hormones, defenses against biotic and abiotic stresses (He et al., 2004; Hong et al., 2008; Leitner et al., 2009; Wilkins et al., 2011; Liu et al., 2013; Yadav et al., 2013). In plants, mitochondria and chloroplasts are organelles that are thought to contribute to NO generation *in vivo* (Galatro et al., 2013; Vanlerberghe, 2013). Although a long standing search for an NO synthase (NOS) in plants similar to NOS enzymes found in mammals has thus far been unsuccessful, suppression of NO signaling in the presence of NOS inhibitors has been reported by several groups, indicating the potential existence of a NOS-like enzyme in plants (Tewari et al., 2013; **Figure 1**).

As a free radical, NO could also react with various intracellular/extracellular targets and form a series of molecules, such as NO radicals (NO^-), nitrosonium ions (NO^+), peroxynitrite ($ONOO^-$), *S*-nitrosothiols (SNOs), higher oxides of nitrogen (NO_x) and dinitrosyl-iron complexes among others, collectively these NO derivatives are termed reactive nitrogen species (RNS; Di Stasi et al., 2002). The functions of RNS, in plant cells are complex because they are implicated in many different physiological processes. *S*-nitrosylation, the covalent attachment of an NO moiety to a reactive cysteine thiol to form an SNO, has emerged as a prototypic redox-mediated modification in plants. For example, *S*-nitrosylation of methionine adenosyltransferase 1 (MAT1; Lindermayr et al., 2006), the *Arabidopsis* type-II metacaspase AtMC9 (Belenghi et al., 2007), PrxII E, a member of the peroxiredoxin family (Romero-Puertas et al., 2007b), non-expression of pathogenesis-related protein 1 (NPR1; Tada et al., 2008), *Arabidopsis thaliana* salicylic acid (SA) binding protein 3 (AtSABP3; Wang et al., 2009), TGACG motif binding factor 1 (TGA1) family (Lindermayr et al., 2010), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase AtRBOHD (Yun et al., 2011), cytoskeletal proteins (Yemets et al., 2011), auxin receptor-transport inhibitor response 1/auxin signaling F-box (TIR1/AFB; Terrile et al., 2011), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Lin et al., 2012) and also *Arabidopsis* histidine phosphotransfer protein (AHP1; Feng et al., 2013) have been reported. These data implies that protein *S*-nitrosylation is a key redox-based modification in plants and a pivotal mechanism to convey NO bioactivity. Peroxynitrite ($ONOO^-$), formed from O_2^- and NO, is also capable of reacting with many classes of biomolecules such as antioxidants and proteins, triggers defense responses in animals and plants (Rubbo et al., 1994a,b). In *Arabidopsis*, $ONOO^-$ could induce hypersensitive response (HR) and defense-related gene expression (Alamillo and Garcia-Olmedo, 2001). Very recently, protein tyrosine nitration, addition of an nitro group (NO_2) to one of the two equivalent ortho carbons of the aromatic ring of Tyr residues and metal nitrosylation, was reported as a new important RNS-mediated post-translational modification (Saito et al., 2006; Astier and Lindermayr, 2012; Tanou et al., 2012; Begara-Morales et al., 2013; Chaki et al., 2013). These findings not only deepen our understanding of NO signaling and function in plants, but

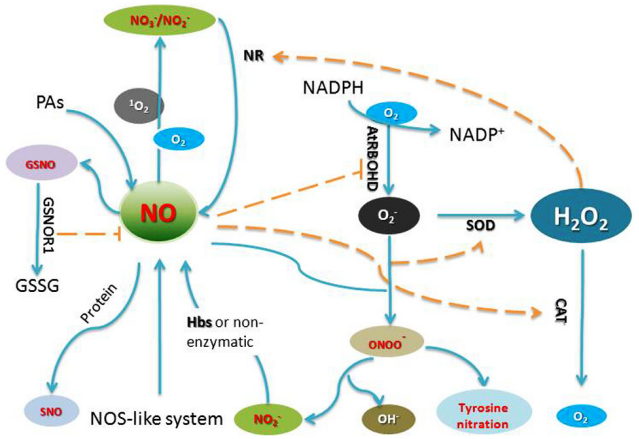

FIGURE 1 | Generation of and crosstalk by RNS and ROS in plant cells. AtRBOHD, an NADPH oxidase; GSNO, *S*-nitrosoglutathione; GSNOR1, *S*-nitrosoglutathione reductase 1; GSSG, glutathione disulfide; NR, nitrate reductase; SOD, superoxide dismutase; Hbs, Hemoglobin; PAs, polyamines; CAT, catalase.

also indicate the existence of RNS cross-talk with other signaling pathways, such as those orchestrated by auxin, cytokinin, SA, jasmonic acid (JA), ethylene (ET), and reactive oxygen species (ROS).

REACTIVE OXYGEN SPECIES

Reactive oxygen species including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radicals (.OH) and singlet oxygen ($^1O_2$) have all been implicated in the control of biological processes in plants. Mitochondria as an "energy factory" are believed to be a major site of ROS production. Alternative oxidase (AOX) has an important influence on both ROS and RNS generation by the respiratory chain in mitochondria (Vanlerberghe, 2013). Peroxisomes are subcellular organelles with an essentially oxidative type of metabolism and produce superoxide radicals (O_2^-) as a consequence of their normal metabolism. Chloroplasts are also a major site of ROS generation in plants (Hideg et al., 2006). The superoxide radicals (O_2^-) and singlet oxygen ($^1O_2$) are produced in chloroplasts by photo-reduction of oxygen and energy transfer from triplet excited chlorophyll to oxygen, respectively (**Figure 1**).

Hydrogen peroxide, a ROS of major biological significance, can form as a result of the reaction of superoxide and also can be generated by specific enzymes (Noctor et al., 2000; Gechev et al., 2006). An oxidative burst, with rapid O_2^- synthesis and its subsequent dismutation to H_2O_2 in the apoplast, is a common response to pathogens, elicitors, wounding, heat, ultra-violet light, and ozone (Orozco-Cardenas et al., 2001; Rao and Davis, 2001). Besides its directly oxidative activity, it is now clear that H_2O_2 has a key signaling role in plants (Gechev et al., 2006; Jiang et al., 2011). H_2O_2 can induce gene expression and modulates signaling proteins, such as protein phosphatases (PP), protein kinases (PK), transcription factors and calcium channels that are located in the plasma membrane or elsewhere (Neill et al., 2002; Lin et al., 2012).

ROS AND NO SIGNALING IN THE HYPERSENSITIVE RESPONSE

A well-documented form of plant programed cell death (PCD) is the HR, characterized by the rapid cell death surrounding infection sites. The HR shows some similarity to the characteristics of animal apoptosis, such as membrane dysfunction, vacuolization of the cytoplasm, chromatin condensation, and endonucleolytic cleavage of DNA (Greenberg and Yao, 2004; Choi et al., 2013; Iakimova et al., 2013). Both NO and ROS have been implicated in controlling the HR process. One of the key determinants for the HR is the balance between intracellular NO and ROS levels (Delledonne et al., 2001; Zaninotto et al., 2006). Following pathogen recognition, NO accumulation occurs concomitant with an oxidative burst, which consists of a biphasic production of apoplastic ROS at the site of attempted invasion (Romero-Puertas et al., 2004). In this context, NO and H_2O_2 are thought to function in combination to promote HR cell death. For example, either of them could cause the release of cytochrome *c* from mitochondria, and affect the caspase-like signaling cascade, leading to the HR (Mur et al., 2006; Tan et al., 2013). Some key components of the defense signaling cascade that are known to be affected by ROS and NO activity include mitogen-activated protein kinases (MAPKs) and phosphatases (**Figure 2**). Thus, modulation of a central MAPK cascade may converge both H_2O_2 and NO signaling pathways activated in response to pathogen infection. In tomato cell suspensions, upon xylanase perception, cells activate a protein kinase pathway required for NO formation and *S*-nitrosylation-dependent mechanisms which are involved in downstream signaling, leading to production of polyamine and ROS production (Lanteri et al., 2011).

Interestingly, many proteins are targets of both NO and H_2O_2 (**Figure 2**). For example, GAPDH that plays a role in mediating ROS signaling in plants is a direct target of H_2O_2 and it is also

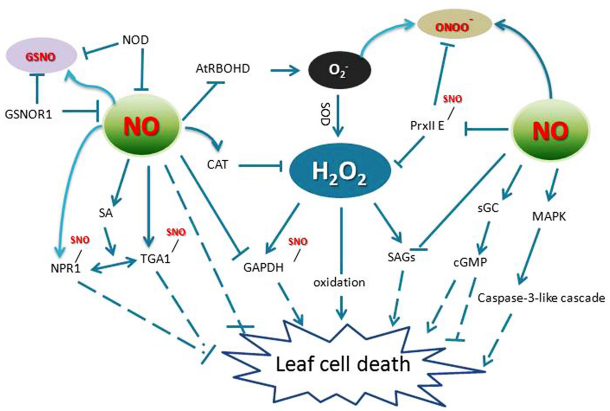

FIGURE 2 | Crosstalk of RNS and ROS in leaf cell death. AtRBOHD, an NADPH oxidase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSNO, *S*-nitrosoglutathione; GSNOR1, *S*-nitrosoglutathione reductase 1; NPR1, non-expression of pathogenesis related protein 1; TGA1, TGACG motif binding factor 1; NR, nitrate reductase; SAG, senescence-associated genes; PrxII E, peroxiredoxin II E; NOD, NO degrading dioxygenase; sGC, soluble guanylate cyclase. MAPK, mitogen-activated protein kinase; SOD, superoxide dismutase; CAT, catalase; cGMP, cyclic guanosine monophosphate; sGC, soluble guanylate cyclase.

a target of NO-mediated *S*-nitrosylation, which blunts its activity (Lindermayr et al., 2005). Also, MAT in mammals is inactivated by H_2O_2 through a reversible and covalent oxidation of a Cys residue. The same Cys residue is also a target for NO, which similarly causes enzyme inactivation (Hancock et al., 2005). Further, PrxII E not only reduces H_2O_2 and alkyl hydroperoxides (Dietz, 2003a,b; Horling et al., 2003), but also functions in detoxifying peroxynitrite. *S*-nitrosylation of PrxII E during the defense response regulates the antioxidant function of this key enzyme and might contribute to the HR (Romero-Puertas et al., 2007a,b; **Figure 2**). As a useful tool to elicit ROS-activated responses, ozone (O_3) has been shown to induce HR-like cell death. During this process, NO accumulation preceded accumulation of ET, JA, SA, and leaf injury, implies that NO is an important signaling molecule in response to O_3 exposure (Rao and Davis, 2001; Ahlfors et al., 2009).

Contrary to its program cell death functions in the HR, NO can also scavenge H_2O_2 and protects plant cells from damage under certain circumstances (Beligni et al., 2002; Crawford and Guo, 2005). NO donors affect both wounding-induced H_2O_2 synthesis and wounding- or JA-induced expression of defense genes (Grun et al., 2006). In *Arabidopsis*, *S*-nitrosoglutathione reductase 1 (GSNOR1) is a key regulator that indirectly controls the global levels of protein *S*-nitrosylation (SNO). Loss-of-function mutations in *GSNOR1* increased total cellular NO and SNO content and compromised both non-host and resistance (*R*) gene-mediated protection and also disabled basal defense responses (Feechan et al., 2005; Wang et al., 2010; **Figure 2**). Further, the mutant *atgsnor1–3* was also perturbed in thermotolerance and resistance to paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride), which induces the production of superoxide and H_2O_2 in wild type leaves (Lee et al., 2008; Chen et al., 2009). Consistent with these results, wild-type plants treated with an NO donor displayed resistance to paraquat (Chen et al., 2009). These studies showed that the *Arabidopsis GSNOR1/HOT5/PAR-2* gene not only regulates SA signaling and thermotolerance by modulating the intracellular SNO level, but also acts downstream of superoxide to regulate cell death.

Interestingly, the increased levels of SNOs in *atgsnor1–3* plants potentiated the HR even in the absence of the cell death agonist SA and apoplastic ROS synthesis. Surprisingly, NO *S*-nitrosylates the NADPH oxidase, AtRBOHD, at Cys890, diminishes its ability to synthesize ROS. This cysteine is also evolutionarily conserved and specifically *S*-nitrosylated in both human and fly NADPH oxidases, suggesting that this mechanism may govern immune responses in both plants and animals (Yun et al., 2011). Thus, NO may control ROS production through protein *S*-nitrosylation to further control the development of cell death processes. Collectively, these findings have provided significant insights into the understanding of the mechanisms underpinning ROS and RNS function in plants, revealing that the ROS/RNS pathway in plant PCD is highly complex and is mediated at least in part by crosstalk with several phytohormone signaling networks.

NO AND ROS CROSSTALK IN LEAF SENESCENCE

Leaf senescence, thought to be another form of plant PCD, is the final stage of leaf development, which is not only controlled by organ age but also triggered by adverse environmental factors (Pourtau et al., 2004; Munns, 2005; Masclaux-Daubresse et al., 2007; Jing et al., 2008; Wu et al., 2012). Additionally, phytohormones such as ET, SA, JA, auxin, ABA, and cytokinins all affect leaf senescence (Lim et al., 2007). In *Arabidopsis*, the level of H_2O_2 increases dramatically in leaf tissue during senescence. In addition to its role in oxidizing macromolecules such as proteins and lipids, H_2O_2 has also been proposed to function as a signal to induce the expression of genes involved in the senescence process (Cui et al., 2013). In agreement with its lower antioxidant capacity, senescent leaf tissue was found to contain elevated levels of ROS. In this context, a number of senescence-associated genes (SAGs) characterized from *Arabidopsis* could be induced by ozone (Miller et al., 1999) and the expression of many other SAGs were also induced by ROS (Navabpour et al., 2003), indicating that ROS might function as a signal to promote senescence. Interestingly, senescence-associated NAC genes (senNACs), key regulators of leaf senescence, were also found to be rapidly and strongly induced by H_2O_2 treatment in both leaves and roots (Balazadeh et al., 2010, 2011). Thus, ROS has a dual role in leaf senescence: to promote the cell death process by directly oxidizing target macromolecules and to drive the expression of senescence-related genes.

Distinct from the positive role of ROS in senescence, NO can both provoke and impede this process, dependent upon its concentration and subcellular location. NO may alleviate the toxicity of ROS and has thus acted as a leaf senescence delaying factor in plants. The NO-deficient mutant *nos1/noa1* showed early leaf senescence (Niu and Guo, 2012) and similarly *Arabidopsis* expressing an NO degrading dioxygenase (NOD) displayed a senescence-like phenotype (Mishina et al., 2007; **Figure 2**). Furthermore, the level of NO is related with the senescence process and is thought to be an essential component involved in plant senescence signaling cascades. In *Arabidopsis* mutant *dnd1*, which lacks a plasma membrane-localized cation channel (CNGC2), early senescence-associated phenotypes (such as loss of chlorophyll, expression level of senescence associated genes, H_2O_2 generation, lipid peroxidation, tissue necrosis, and SA levels) were all elevated relative to wild type. Basal levels of NO in *dnd1* leaves were lower than wild type, suggesting that the function of CNGC2 may impact downstream "basal" NO production in addition to its role linked to NO signaling (Ma et al., 2010). NO generation is therefore thought to act as a negative regulator during plant leaf senescence signaling. The protective effect of NO against ROS induced cell death can also be linked to the enhanced activity of antioxidant enzymes, as negative regulator of the chlorophyll catabolic pathway and as drivers for positively maintaining the stability of thylakoid membranes during leaf senescence (Liu and Guo, 2013).

On the other hand, NO can also promote the leaf senescence. *Arabidopsis* AtFer1, one of the best characterized plant ferritin isoforms to date, strongly accumulates upon treatment with excess iron, via an NO-mediated pathway. The AtFer1 isoform is functionally involved in events leading to the onset of age-dependent senescence in *Arabidopsis* and its iron-detoxification function during senescence is required when ROS accumulates (Murgia et al., 2007). Recently identification of an NO accrual mutant *noe1* (*nitric oxide excess 1*) in rice revealed that *NOE1* encoded a rice catalase (CAT) OsCATC. Interestingly, *noe1* plants exhibited an

increase of H_2O_2 in their leaves, which consequently promoted NO production via activation of nitrate reductase. Removal of excess NO reduced cell death in both leaves and suspension cultures derived from *noe1* plants, implicating that NO acts as an important endogenous mediator of H_2O_2-induced leaf cell death. Reduction of intracellular SNO levels, generated by overexpression of OsGSNOR alleviated leaf cell death in *noe1* plants. Thus, *S*-nitrosylation was also involved in light-dependent leaf cell death in *noe1*. Collectively, these data suggest that both NO and SNOs are important mediators in the process of H_2O_2-induced leaf cell death in rice (Lin et al., 2012; Wang et al., 2013). OsGSNOR in *noe1* plants reduced SNO levels, consistent with a key role for this enzyme in SNO homeostasis. Moreover, the results show that no change in H_2O_2 content occurred in either *GSNOR*-overexpressing or *GSNOR*-RNAi transgenic lines in the context of *noe1* background, suggesting that NO might function downstream of H_2O_2 in a light-driven leaf cell death in rice. It was found that NO treatment led to rapid cell death and induced H_2O_2 accumulation in maize leaves, and pharmacological studies also suggested that NO-induced cell death is in part mediated via H_2O_2, therefore H_2O_2 may be involved in NO-induced cell death in maize leaves (Kong et al., 2013). These discrepancies for the role of NO in cell death might be due to the differences in plant species, redox state, and growth conditions. Both NO and H_2O_2 could induce leaf cell death during which they could crosstalk with each other through different pathways.

NO AND ROS IN OTHER TYPES OF PLANT CELL DEATH

Some reports also describe the cross-talk of NO and ROS in other kinds of cell death in plants. Gibberellin (GA)-induced PCD in barley (*Hordeum vulgare* cv. Himalaya) aleurone layers is mediated by ROS and NO is a protective antioxidant. NO donors delay this PCD process, but do not inhibit metabolism in general, or the GA-induced synthesis and secretion of alpha-amylase. The amounts of CAT and superoxide dismutase (SOD) are greatly reduced in aleurone layers treated with GA. Treatment with GA in the presence of NO donors delays the loss of CAT and SOD. Thus, NO may be an endogenous modulator of PCD in barley aleurone cells (Beligni et al., 2002). Furthermore, the exogenous application of NO rendered the plants more tolerant to arsenic (As)-induced oxidative damage by enhancing their antioxidant defense and glyoxalase system (Hasanuzzaman and Fujita, 2013). Previous work has also shown that NO acts as a pivotal positive mediator in cadmium (Cd)-induced PCD in suspension cell cultures. NO strongly counteracts Cd-induced ROS mediated cytotoxicity in *Brassica juncea* by controlling antioxidant metabolism (De Michele et al., 2009; Verma et al., 2013). Similarly, a role for NO as an antioxidant during heavy metal mediated toxicity has been highlighted recently by Saxena and Shekhawat (2013).

On the other hand, NO could also aid ROS-induced PCD. In pollen-pistil interactions, self-incompatibility (SI) induces relatively rapid and transient increases in ROS and NO. As ROS/NO scavengers alleviated both the formation of SI-induced actin punctate foci and also activation of a DEVDase/caspase-3-like activity (Wilkins et al., 2011). In tobacco BY-2 cells, sphinganine or dihydrosphingosine (d18:0, DHS) induce a calcium dependent PCD and trigger H_2O_2 production via the activation of NADPH oxidase(s). They also promote NO production, which is required for cell death induction (Da Silva et al., 2011). NO accumulated in Cd-induced PCD and promoted Cd-induced *Arabidopsis* PCD by promoting MPK6-mediated caspase-3-like activation (Ye et al., 2013). So the different roles of RNS in PCD and their crosstalk with ROS depend on the plant species, growth conditions and redox status.

CONCLUSION

In plants, RNS and ROS synthesis is a routine requirement for cells to undergo PCD, these small molecules can act either synergistically or independently (Clarke et al., 2000; Orozco-Cardenas and Ryan, 2002; Bright et al., 2006). The accumulating data suggests significant cross-talk occurs between RNS and ROS (**Figure 1**), although the clear relationship of RNS and ROS in the process of cell death remains elusive. NO and ROS could regulate the synthesis each other. During HR, NO can affect ROS synthesis through *S*-nitrosylating NADPH oxidase AtRBOHD (Yun et al., 2011). On the other hand, in rice *noe1* mutant, in the absence of OsNOE1/OsCATC function, the accumulation of H_2O_2 induces NO production through elevating nitrate reductase expression, which is further integral to H_2O_2 induced leaf cell death through *S*-nitrosylation of GAPDH and thioredoxin (Lin et al., 2012; Wang et al., 2013). Cross-talk of NO and H_2O_2 is a prominent feature in the activities of these small molecules. RNS and ROS also play important roles in modulating the activity of target proteins. A complete list of signaling pathways regulated by ROS or RNS still awaits identification, the data presented in this review are therefore far from offering a comprehensive picture of the function of NO and ROS during plant PCD. Thus, further work is needed to understand how these key molecules trigger the onset and development of plant cell death.

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Limits in the use of cPTIO as nitric oxide scavenger and EPR probe in plant cells and seedlings

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Over the last decade the importance of nitric oxide (NO) in plant signaling has emerged. Despite its recognized biological role, the sensitivity and effectiveness of the methods used for measuring NO concentration in plants are still under discussion. Among these, electron paramagnetic resonance (EPR) is a well-accepted technique to detect NO. In the present work we report the constraints of using 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) in biological samples as spin trap for quantitative measurement of NO. EPR analyses on *Arabidopsis* cell cultures and seedlings show that cPTIO(NNO) is degraded in a matter of few minutes while the (INO) compound, produced by cPTIO and NO reaction, has not been detected. Limitations of using this spin trap in plant systems for quantitative measurements of NO are discussed. As NO scavenger, cPTIO is widely used in combination with 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) fluorescent dye in plant research. However, the dependence of DAF-FM fluorescence on cPTIO and NO concentrations is not clearly defined so that the range of concentrations should be tightly selected. In this context, a systematic study on cPTIO NO scavenging properties has been performed, as it was still lacking for plant system applications. The results of this systematic analysis are discussed in terms of reliability of the use of cPTIO in the quantitative determination and scavenging of NO in plants and plant cultured cells.

Keywords: plant, nitric oxide, cPTIO, electron paramagnetic resonance (EPR), NO scavenger, DAF-FM, *Arabidopsis*

INTRODUCTION

Nitric oxide (NO) is a signal molecule involved in controlling both physiological processes and stress responses (Mur et al., 2013). It plays an important role in root organogenesis and development (Correa-Aragunde et al., 2004) and in auxin signaling (Kramer and Bennett, 2006) and perception (Terrile et al., 2012). In response to pathogen attacks, NO turns to be a key molecule in the hypersensitive response (HR) and programmed cell death (PCD) events (Wang et al., 2013). Recently, the role of NO has also been investigated in abscisic acid (ABA)-associated response of guard cells to pathogens (Ye et al., 2013).

The central role of NO in plants is corroborated by the presence of many different enzymatic and non-enzymatic sources (Gupta et al., 2010). However, the controversial existence of NO synthase-like enzymes makes it difficult to define the specific NO source engaged in a specific physiological process and to understand how it is involved in it. For this reason, in order to establish whether and where NO is produced by specific cells and tissues, plant researchers rely on several indirect methods of analysis. Many of the methods developed for NO detection capitalize on its high diffusibility as well as on its broad spectrum of chemical reactivity. However, in biological systems, the use of these methods is limited by the short half-life of the molecule (Woldman et al., 1994; Gupta and Igamberdiev, 2013).

Electron paramagnetic resonance (EPR) is a well-accepted spectroscopic technique to detect NO in a liquid phase (Hogg, 2010). This technique is selective for monitoring radical species. In principle, being NO a radical, a direct measurement by EPR should be possible; however, due to its fast spin relaxation time, it cannot be detected. Therefore, the methods of NO detection in solution through EPR are based on the trapping of NO with the formation of stable paramagnetic species (Hogg, 2010). As a matter of fact, in biological samples spin trapping methods are largely used for detection of short-living radicals such as O_2^-, $OH^\bullet$, both *in vivo* and *in vitro* (Berliner, 2000). Spin trapping is necessary since conventional EPR requires a steady state concentration of the free radical higher than 0.01 μM.

Iron dithiocarbamates have been widely used as spin traps, due to their high affinity for NO. The formation of stable nitrosyl iron-dithiocarbamate complexes gives a three-line EPR spectrum at room temperature, characterized by the hyperfine interaction with the N nucleus of NO (Vanin et al., 2000). However, the use of iron dithiocarbamates is problematic for quantitative NO determination, either *in planta* or in cultured cells, due to the interference of nitrites and nitrates that can produce NO under the reducing experimental conditions required for this assay (Hogg, 2010).

Alternatively, nitroxide spin traps have been tested *in vitro* and in animal cell systems (Haseloff et al., 1997). A well-known nitroxide spin trap for NO used in biological

samples is 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) that belongs to the nitronyl nitroxides (NNO) compounds. NNOs are stable organic radicals that react with NO, with rate constant of about 10^4 M^{-1} s^{-1}, forming imino nitroxides (INO) with a significant change in the associated EPR spectra (Yoshioka et al., 1996). In fact, following this reaction, the number of lines in the EPR spectra changes from five to seven.

For its chemical properties cPTIO has been commonly used also as a NO scavenger in combination with 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) fluorescent dye, although many pitfalls have been evidenced (Vitecek et al., 2008; Rumer et al., 2012). Conversion of DAF-FM to the corresponding triazole forms (DAF-FM-T) by reaction with NO causes little changes in the absorbance maxima but greatly increases the fluorescence quantum efficiency. DAF-FM dyes react with N_2O_3, a by-product of NO oxidation, with a resulting increase in fluorescence, dependent on NO concentration. cPTIO is used as a scavenger of NO, to remove the increase of DAF-FM fluorescence, and prove in this way the production of NO in the system. However, it has also been shown that cPTIO, under particular experimental conditions, may facilitate formation of N_2O_3 by increasing the rate of NO oxidation, thus inducing an increase, instead of a decrease, of DAF-FM fluorescence (Arita et al., 2006). In fact, cPTIO oxidizes NO forming $^{\bullet}NO_2$ radical ($NO + cPTIO \rightarrow {}^{\bullet}NO_2 + cPTI$), which in turn can react with NO to form N_2O_3 ($NO_2 + NO \rightarrow N_2O_3$). The sensitivity of the fluorescence intensity to pH and ascorbic acid was also considered as a source of uncertainty in the detection of NO in plants.

Despite these intrinsic problems, the advantages of cPTIO to be specific for NO and cell permeable (Vitecek et al., 2008), along with its widespread use in plant experiments, prompted us to perform a systematic study on cPTIO NO scavenging properties, since a detailed analysis was still lacking regarding applications to plant systems.

RESULTS

cPTIO AS A SPIN TRAP FOR NO DETECTION IN PLANTS

We have evaluated the use of cPTIO as NO spin trap in plants by analyzing its EPR spectrum in different experimental conditions. In **Figure 1**, the reference spectrum of 100 μM cPTIO(NNO) in water is shown. Based on the stoichiometry of the reaction between cPTIO and NO (1:2) (Hogg et al., 1995) and on the NO release stoichiometry by the NO donor ((Z)-1-(*N*-Methyl-*N*-[6-(*N*-methylammoniohexyl)-amino])-diazen-1-ium-1,2-diolate) MAHMA NONOate, 200 μM of the NO donor was used to obtain a saturated signal corresponding to about 100 μM cPTIO(INO), whose EPR spectrum is also shown in **Figure 1**. In line with previous literature, cPTIO(NNO) gives a five-line EPR spectrum, characterized by hyperfine splitting due to the presence of two equivalent N nuclei, while cPTIO(INO) shows the specific seven-peak spectrum due to the presence of two non-equivalent N nuclei.

To assess the spin trap stability in the presence of biological samples, a series of experiments were performed *in vivo* on *Arabidopsis* cultured cells, by incubating 5-day-old cell cultures with 100 μM cPTIO(NNO) or cPTIO(INO). The EPR measurements were done on the culture medium after different incubation times (from 1 to 130 min). It was observed that the intensity of EPR signals of both cPTIO(NNO) and cPTIO(INO) rapidly decreased in the first minutes of incubation, reaching nearly zero after 130 min (**Figure 2**). The disappearance of cPTIO(NNO) signal was not followed by the appearance of cPTIO(INO) spectrum.

In order to verify whether the reduction of cPTIO EPR signal was associated with the presence of a cell-linked activity, EPR measurements were performed incubating cPTIO(NNO) either in exhausted culture medium, withdrawn from 5-day-old cell cultures, or in the presence of boiled 5-day-old cell cultures. In both cases, the intensity of EPR signals was maintained for longer time compared with the previous experiments, with a signal decrease of less than 10% after 180 min (**Figure 3**).

Two hypotheses can explain why the intensity of EPR signals rapidly decreases in cell cultures. The first is a fast uptake of cPTIO, which accumulates inside the cells, so that it becomes not measurable in the culture medium; the second is that cPTIO is rapidly transformed in an EPR silent product, either inside, after uptake, or outside the cells.

To clarify this point, 100 μM cPTIO was incubated with *Arabidopsis* cell cultures for 10 min. After this time, the cells were separated from the medium and the EPR signal was measured both in the medium and in the total soluble cell extract. The intensity of the EPR signal measured in the external medium significantly decreased after 10 min of incubation (**Figure 4**). A

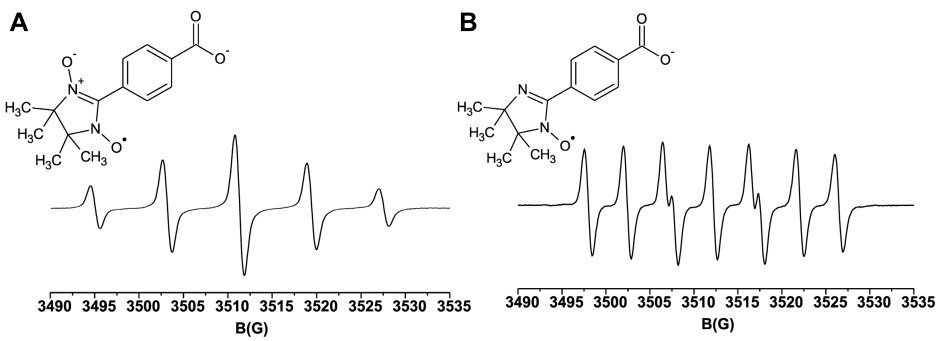

FIGURE 1 | Molecular structures and room temperature EPR spectra. (A) 100 μM cPTIO(NNO); **(B)** 100 μM cPTIO(INO), in water.

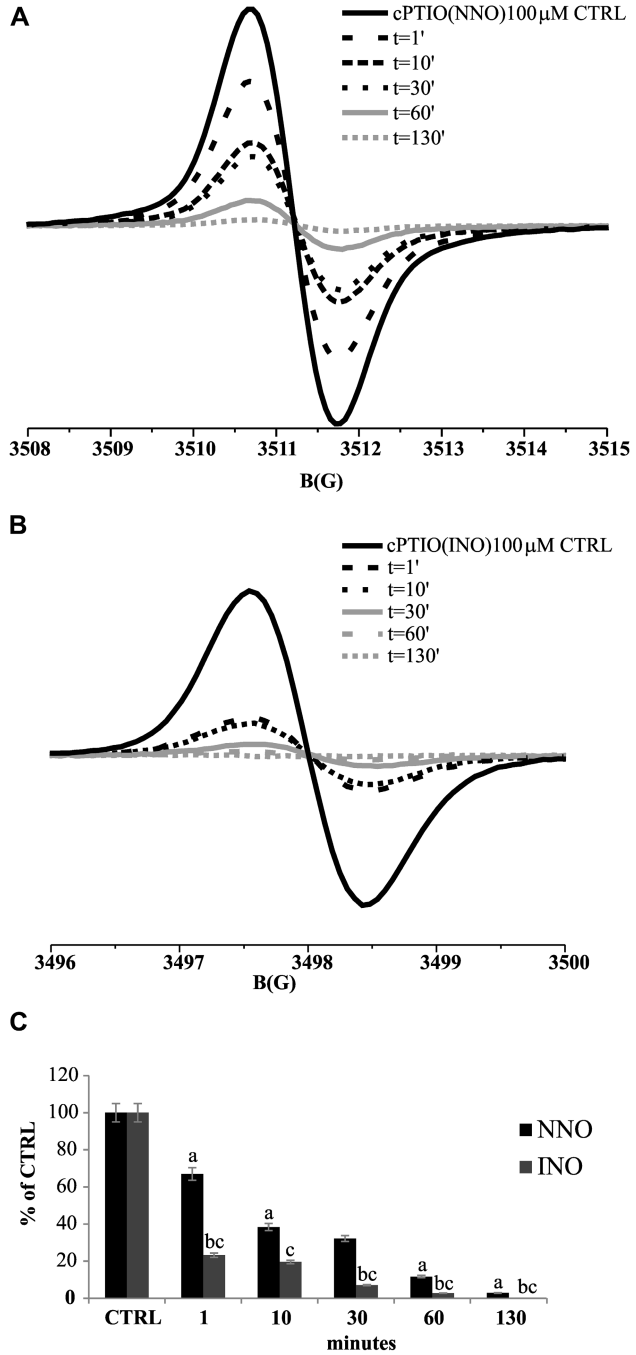

FIGURE 2 | Time dependence of cPTIO(NNO) and cPTIO(INO) EPR signals in suspension cultured cells. Central line of cPTIO(NNO) **(A)**, and low field, first line of cPTIO(INO) **(B)** EPR spectra were quantified. In **(C)** the intensity of EPR signal for each measurement, is presented as percentage of the total signal resulting from measurement of 100 μM cPTIO(NNO) or (INO) dissolved in water (CTRL) ± SD. cPTIO(NNO) or (INO) was added to the supernatant of 5-day-old *Arabidopsis* suspension cultured cells and aliquots of the medium were collected at the time points indicated. A Student's *t*-test was performed for each experiment and statistically significant data are marked: (a) $p < 0.01$ cPTIO(NNO) compared with the previous time point, (b) $p < 0.01$ cPTIO(INO) compared with the previous time point, (c) $p < 0.01$ cPTIO(INO) compared with cPTIO(NNO) at the same time point.

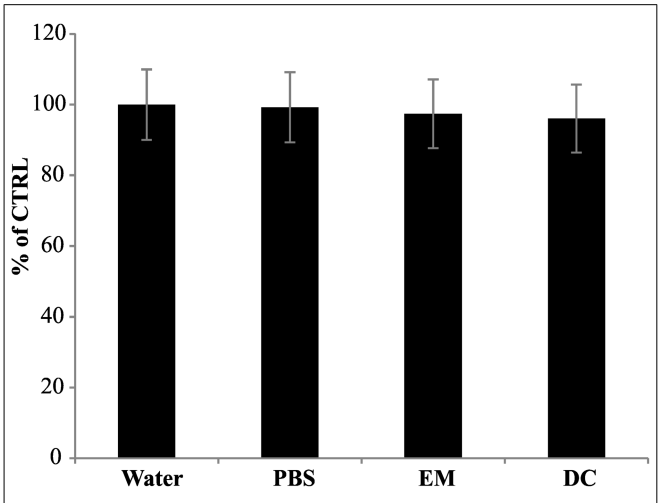

FIGURE 3 | Electron paramagnetic resonance signal of cPTIO incubated in water, PBS, exhausted medium or with dead cells. cPTIO(NNO) was incubated in water, PBS, exhausted medium (EM) or with boiled dead cells (DC). EPR spectra of the samples were detected after 180 min of incubation. Intensities of EPR signals are given as percentage of the total signal at t_0.

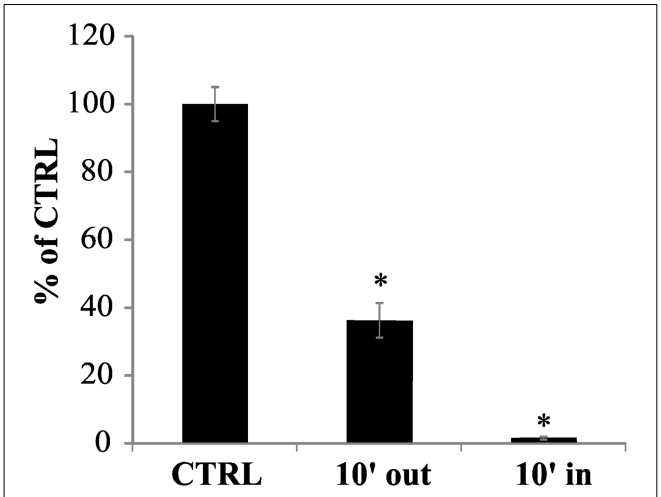

FIGURE 4 | cPTIO uptake in cell cultures. cPTIO(NNO) was incubated with cell cultures. At 10 min a sample from the culture medium was collected (10′ out) and all cells were harvested. The cells were disrupted and centrifuged. An aliquot of the supernatant was collected (10′ in). EPR signals were detected and given as percentage of the total signal resulting from measurement of 100 μM cPTIO(NNO) dissolved in water (CTRL) ± SD. A Student's *t*-test was performed and samples statistically different from the CTRL marked with an asterisk

small cPTIO(NNO) signal was detected also in the cell extract, showing that cPTIO was actually entering the cells but its concentration resulted strongly reduced when compared to the bulk concentration initially added to the sample (about 1% of the signal of 100 μM cPTIO in water; **Figure 4**). This result proves that the decrease of the EPR signal observed in the medium is not due to the spin trap accumulation inside the cells, but rather to the disappearance of cPTIO.

The degradation of cPTIO(INO) by the cells, at a faster rate compared to that of cPTIO(NNO) (**Figure 2**), implies that, for an *in vivo* quantitative measurement of NO via EPR, the use of cPTIO is not feasible because cPTIO(INO) is not stable and does not accumulate in a steady state concentration reaching the sensitivity of the EPR technique. On the other hand, the fact that 100 μM cPTIO(NNO) disappears in a short time (with a decay time constant of about 15 min^{1}) and in a measurable way indicates that the endogenous NO, present in low concentration, is not the main responsible for the reactions undergone by cPTIO(NNO). This hypothesis was also supported by a series of experiments on cultured cells treated with salicylic acid (SA), which induces an increase of NO production (Zottini et al., 2007), to evaluate influence of NO on the decay rate of cPTIO(NNO) EPR signal. In that instance, it was found that the decay rate of cPTIO(NNO) was not affected by the treatment (not shown) meaning that the main reason for cPTIO(NNO) disappearance was not the reaction with NO but with other substrates such as reductans present in the cells (Haseloff et al., 1997). Thus, both the fast transformation of cPTIO(INO) and the competitive reactions of cPTIO(NNO) with substrates others than NO contribute to hinder the quantification of NO in living cells by using cPTIO as spin trap.

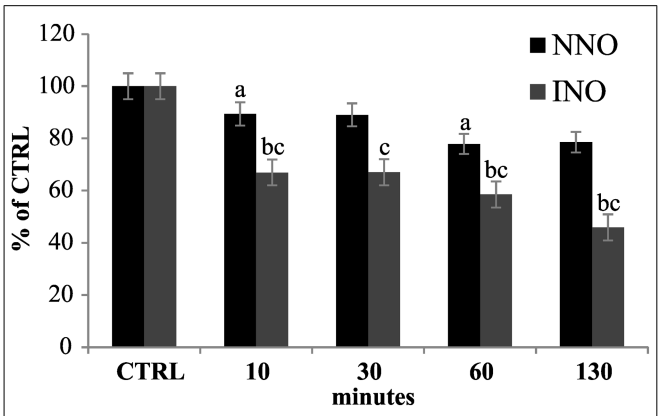

FIGURE 5 | Time dependence of cPTIO EPR signals in seedlings. 8-day-old *Arabidopsis* seedlings were incubated in 50 ml of liquid culture medium. cPTIO(NNO) or (INO) was added to the supernatant and aliquots of the medium were taken at indicated time points. EPR measurements are presented as percentage of the total signal resulting from measurement of 100 μM cPTIO(NNO) and (INO) dissolved in water (CTRL) ± SD. A Student's *t*-test was performed for each experiment and statistically significant data marked: (a) $p < 0.01$ cPTIO(NNO) compared with the previous time point, (b) $p < 0.01$ cPTIO(INO) compared with the previous time point, (c) $p < 0.01$ cPTIO(INO) compared with cPTIO(NNO) at the same time point.

cPTIO NO SCAVENGING EFFICACY IN *IN VIVO* MEASUREMENTS

cPTIO is widely used as NO scavenger in plant experimental systems to validate the involvement of NO in pathways triggered by different external/internal stimuli. cPTIO is used in plant cell cultures but also in experiments carried out on plant seedlings. Therefore, the kinetics of cPTIO reactions was also examined in this experimental system. The experiments were performed on *Arabidopsis* 8-day-old seedlings, incubated in 50 ml of liquid medium, by adding 100 μM cPTIO(NNO) to the external medium. The EPR measurements were performed on the culture medium after several incubation times (from 1 to 130 min). As shown in **Figure 5**, a decrease of EPR signal associated to cPTIO was observed, but it was slower when compared to that of cell cultures. A possible explanation for different decreasing rates could be the much more complex and slower process of cPTIO uptake in the whole plant compared to cultured cells. Thus, the uptake may become a rate-determining step in the cPTIO EPR signal disappearance. The decrease in the EPR signal of cPTIO was not accompanied by the formation of the INO EPR signal in cell cultures, as well as in seedlings.

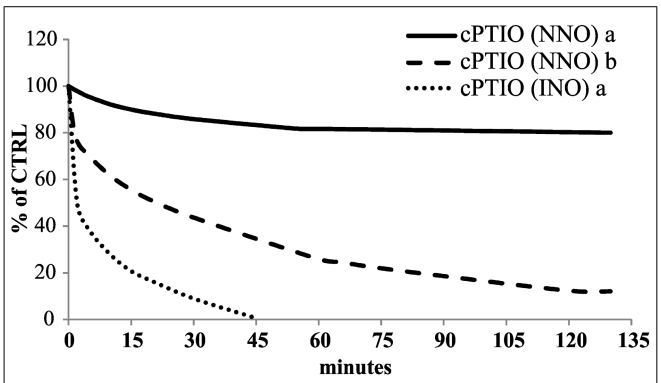

FIGURE 6 | Time dependence of cPTIO EPR signals in *Arabidopsis* total soluble extract. 100 μM cPTIO(NNO) or (INO) was incubated in 50 mM PBS pH 7 in the presence of a protein concentration of (a) 0.3 mg/ml or (b) 1.8 mg/ml *Arabidopsis* total extract. The time course of the reactions was followed as decrease of the EPR signals. The EPR signals for each measurement were presented as percentage of the total signal resulting from measurement of 100 μM cPTIO dissolved in PBS. The plot reported is representative of three independent experiments.

The experiments performed on boiled cell culture reported above indicated that the disappearance of the cPTIO EPR signal was dependent on a cell-linked activity. To validate this hypothesis the stability of cPTIO incubated with different amount of *Arabidopsis* total soluble extract was investigated. **Figure 6** shows the time dependence of the 100 μM cPTIO(NNO) and (INO) EPR signals following the addition of different amounts of extract. The intensity of EPR signals strongly decreased depending on both the incubation time and the extract concentration. This result strongly supports an enzyme-dependent transformation of the chemical compounds. cPTIO(INO) showed a faster decay rate compared to cPTIO(NNO), using the same concentration of total extract. Moreover the EPR signal of cPTIO(INO) in the presence of the higher concentration of total soluble extract (1.8 mg/ml) was not even detectable (data not shown).

A common method for NO detection is the use of DAF-FM fluorescent dye and its membrane-permeable diacetate form (Kojima et al., 1998). DAF-FM has been used to localize NO production site in plant cells and tissues (Correa-Aragunde et al., 2004), and quantify the production of NO in suspension cultured cells (Krause and Durner, 2004). In each of these studies, fluorescence quenching by cPTIO has been used as confirmation that DAF-FM fluorescence was indeed due to NO (Gupta and Igamberdiev, 2013),

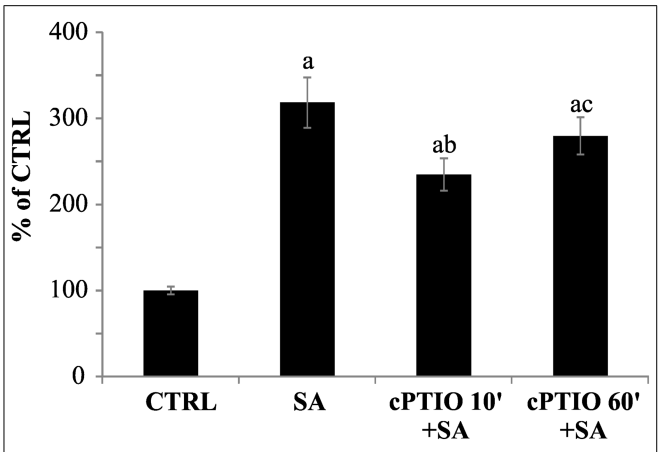

FIGURE 7 | Time dependence of DAF-FM fluorescence quenching by cPTIO. 100 μM cPTIO(NNO) was added to cell cultures and kept for different incubation times (10 or 60 min) before 1 mM salicylic acid (SA) addition. NO levels were analyzed 60 min after SA treatment by using DAF-FM (excitation: 488 nm, detection: 515 nm). Signal from 3D reconstruction was quantified by image densitometry and reported as percentage of the not treated sample (CTRL) ± SD. A Student's *t*-test was performed for each experiment and statistically significant data marked: (a) $p < 0.01$ compared with CTRL, (b) $p < 0.01$ compared with SA, (c) $p < 0.01$ compared with cPTIO 10′+SA.

since cPTIO is known to be acting as a specific NO scavenger. However, it has been shown that in the presence of high levels of NO, cPTIO can induce an increase of DAF-FM fluorescence, rather than a quenching, through a complex pathway of oxidation reactions (Vitecek et al., 2008).

The experimental data reported here have proven that cPTIO is rapidly transformed in an EPR silent compound in samples containing cells or seedlings. Thus, it is important to understand whether the reaction products of cPTIO are still able to scavenge NO. To evaluate this, cell cultures stimulated by SA were pretreated for different incubation times with cPTIO, and NO was detected by DAF-FM. In a previous paper (Zottini et al., 2007), NO production induced by SA in *Arabidopsis* cell cultures has been already reported and in that case it was measured with DAF-FM and oxyhemoglobin, in parallel. The two techniques showed indeed comparable results, confirming NO production triggered by SA.

As reported in **Figure 7**, the increasing of cPTIO incubation time leads to a reduction of its scavenging efficacy. While cPTIO pre-incubated for 10 min is able to scavenge SA-induced NO, a longer pre-incubation significantly decreases the scavenging efficacy. These results demonstrate that molecules deriving from cPTIO cell reactions are not able to scavenge NO.

DISCUSSION

In the present work, we provide a systematic study to evaluate the efficacy of using cPTIO as NO spin trap and NO scavenger in plant systems, in particular, in cell cultures and seedlings.

The nitronyl nitroxides have been already used *in vitro* and in animal systems as spin traps for NO (Woldman et al., 1994; Haseloff et al., 1997) because of their specificity for NO compared to other spin traps, such as iron dithiocarbamates or oxyhemoglobin (Hogg, 2010).

It has been reported that in those systems cPTIO(NNO) and (INO) are transformed in the EPR silent form hydroxylamine (Woldman et al., 1994; Haseloff et al., 1997). The occurring reaction is likely a reduction associated to the presence of reducing substrates, such as glutathione and/or ascorbate, in the cell environment. Superoxide has also been reported as a possible reductant of nitroxides (Haseloff et al., 1997).

Our results strongly suggest that, also in plant cells, different reducing species may react with cPTIO. Therefore, the use of nitronyl nitroxides as spin traps for NO detection via EPR in plant systems, where endogenous rates of NO generation are very low, is compromised by their very rapid reduction into diamagnetic EPR silent products.

We also evidenced that the reduction of cPTIO is an enzyme-mediated process. In fact, it was observed that cPTIO(NNO) and cPTIO(INO) EPR signals did not decrease in fresh culture medium (data not shown), in exhausted medium, or in boiled cell suspensions. As expected, their decay rates increased after the addition of cellular extract.

Summarizing, the competitive reactions of cPTIO, and the fast reduction of cPTIO(INO), make the use of cPTIO as spin trap for NO detection via EPR unmanageable, at least in the micromolar range of NO concentrations.

The other question addressed was whether the use of cPTIO as NO scavenger was reliable, in spite of all the occurring transformation events. To shed light on this controversial point, we carried out a series of experiments with plant cells and seedlings. The results clearly indicate that also the scavenging abilities of cPTIO may be impaired due to cellular reactions. Actually, we observed that when NO production was induced by SA, the scavenging efficacy of 100 μM cPTIO was significantly reduced in a time-dependent manner. We, thus, infer that to obtain a strong scavenging effect, a higher concentration of cPTIO should be used. On the other hand, it has to be kept into consideration that high concentrations of cPTIO can give rise to artifacts, when DAF-FM is used as detection method (Arita et al., 2006).

In conclusion, the reported analysis underlines the drawbacks of using cPTIO as EPR probe for *in vivo* measurements of NO in plants. In addition, the results provide helpful indication for the right use of cPTIO as NO scavenger. In fact, in order to effectively scavenge NO, the parallel depletion of cPTIO in living cells has to be taken into account. The relatively low cPTIO concentration used in our experiments has allowed us to evidence better the time dependence of cPTIO degradation, confirming data obtained by EPR measurements. At the same time, it is evidenced that the use of low concentration of cPTIO could compromise its scavenging efficacy due to competitive reactions.

The complex chemical behavior of cPTIO in plant environment may explain why cPTIO is not always able to completely scavenge NO, especially for treatments inducing a gradual and continuous production of NO.

Since cPTIO is highly specific to NO, its use remains valuable. However, to produce significant data, and observe the scavenging effect of cPTIO, concentrations and incubation time should be accurately chosen, depending on the analyzed system and in relation to the amount of NO produced.

MATERIAL AND METHODS

CHEMICALS

2-4-Carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (Alexis Biochemicals ALX-430-001), DAF-FM-DA (Alexis Biochemicals, ALX-620-071), SA (S7401 SIGMA), MS medium salt including vitamins (Duchefa M 0409), MAHMA NONOate (Alexis, Vinci, Italy).

CELL CULTURES

Suspension cell culture was generated from hypocotyls dissected from young plantlets of *Arabidopsis* (ecotype *Landsberg erecta*) and subcultured in AT3 medium (Desikan et al., 1996). For subculture cycles, 5 ml of cell culture volume [0.8 g fresh weight (FW)] was placed in 100 ml Erlenmeyer flasks containing 45 ml of liquid medium. Cells were subcultured in fresh medium at 7 days intervals and maintained in a climate chamber on a horizontal rotary shaker (80 rpm) at 24°C with a 16-/8-h photoperiod and a light intensity of 70 mmol m^{-2} s^{-1}. All analyses and treatments with filter-sterilized solutions of SA were carried out with 5-day-old cultures (4 g FW).

Arabidopsis SEEDLINGS

Seeds of *Arabidopsis* (ecotype *Columbia*) were surface sterilized by washing with 70% EtOH, 0.05% Triton X 100. After the sterilization they were grown on MS – ½ medium supplemented with 0.5 g/l MES-KOH pH 5.7, 0.8% plant agar, and 1% sucrose. After 48 h of incubation at 4°C in the dark, plates were put in a growing chamber at 22°C and long day light period (16 h light/ 8 h dark). The plates were kept vertically. Seedlings of 8 days were used for the experiments (4 g FW).

Arabidopsis TOTAL SOLUBLE EXTRACT

100 mg of *Arabidopsis* cells or seedlings were homogenized by Eppendorf micropestle in extraction buffer added 1:1 w/v (50 mM PBS pH 7, EDTA 1 mM, protease inhibitor cocktail). The samples were centrifuged 1 min at 16000 × *g* at 4°C. The supernatant was recovered and quantified by Bradford protein assay test (Biorad). A protein content of 0.3 or 1.8 mg/ml was used in each experiment.

cPTIO ANALYSES

100 μM cPTIO(NNO) or cPTIO(INO) was added directly to the *Arabidopsis* cells culture. Aliquots of the medium were collected at different incubation time, and immediately frozen in liquid nitrogen. Samples were then analyzed by EPR spectroscopy at room temperature, after thawing.

The experiments with boiled dead cells were performed using *Arabidopsis* cell cultures, boiled for 30 min.

The experiments with exhausted medium were performed incubating 100 μM cPTIO(NNO) in the medium withdrawn from 5-day-old cell cultures.

8 days-old *Arabidopsis* seedlings (4 g FW) were incubated in 50 ml liquid culture medium (MS – ½ medium supplemented with 0.5 g/l MES-KOH pH 5.7, 0.8% plant agar, and 1% sucrose). 100 μM cPTIO was added to the medium. Aliquots of the medium were analyzed by EPR.

100 μM cPTIO was incubated with 0.3 or 1.8 mg/ml total soluble extract concentration, diluted in PBS and added to the capillary for EPR measurements.

The EPR signals for each measurement were presented as percentage of the total signal resulting from measurement of 100 μM cPTIO dissolved in water.

STATISTICAL ANALYSES

All experiments were performed at least three times on independent biological replicates. The results are presented as mean ± SD (standard deviation). Statistical differences were determined by using Student's *t*-test. Statistical significance was assigned at $p < 0.01$.

DAF-FM ANALYSES

2-4-Carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide was added to different flasks of *Arabidopsis* cell culture of 5 days (4 g FW) and incubated for different pre-incubation times. After the cPTIO pre-incubation 15 μM DAF-FM-DA was loaded in the cells as previously described (Zottini et al., 2007). 1 mM SA was added to the cell culture and cells were analyzed after 60 min of treatment. Samples were observed by confocal microscopy using the 488 Argon line for excitation. 3D reconstruction of the cells were obtained by Nikon PCM2000 (Biorad) laser scanning confocal microscope. DAF-FM Fluorescence was quantified by image densitometry analysis of the pixel intensities using ImageJ software (NIH, USA). At least 20 cells per samples were singularly analyzed.

EPR SPECTROSCOPY

Room temperature continuous wave EPR spectra were collected using a Bruker Elexsys E580-X-band spectrometer equipped with the Elexsys Super High Sensitivity Probehead. All measurements were performed in capillaries (ID 0.9 mm; 50 μl total volume). Acquisition parameters were the following: microwave frequency = 9.86 GHz; modulation amplitude in the range 0.15–0.3 Gauss, microwave power = 6.370 mW; sweep time 167.77 s, time constant 40.96 ms.

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RuBisCO depletion improved proteome coverage of cold responsive S-nitrosylated targets in Brassica juncea

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Although in the last few years good number of S-nitrosylated proteins are identified but information on endogenous targets is still limiting. Therefore, an attempt is made to decipher NO signaling in cold treated *Brassica juncea* seedlings. Treatment of seedlings with substrate, cofactor and inhibitor of Nitric-oxide synthase and nitrate reductase (NR), indicated NR mediated NO biosynthesis in cold. Analysis of the *in vivo* thiols showed depletion of low molecular weight thiols and enhancement of available protein thiols, suggesting redox changes. To have a detailed view, S-nitrosylation analysis was done using biotin switch technique (BST) and avidin-affinity chromatography. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is S-nitrosylated and therefore, is identified as target repeatedly due to its abundance. It also competes out low abundant proteins which are important NO signaling components. Therefore, RuBisCO was removed (over 80%) using immunoaffinity purification. Purified S-nitrosylated RuBisCO depleted proteins were resolved on 2-D gel as 110 spots, including 13 new, which were absent in the crude S-nitrosoproteome. These were identified by nLC-MS/MS as thioredoxin, fructose biphosphate aldolase class I, myrosinase, salt responsive proteins, peptidyl-prolyl cis-trans isomerase and malate dehydrogenase. Cold showed differential S-nitrosylation of 15 spots, enhanced superoxide dismutase activity (via S-nitrosylation) and promoted the detoxification of superoxide radicals. Increased S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase sedoheptulose-biphosphatase, and fructose biphosphate aldolase, indicated regulation of Calvin cycle by S-nitrosylation. The results showed that RuBisCO depletion improved proteome coverage and provided clues for NO signaling in cold.

Keywords: S-nitrosylation, thiol pool, nitric oxide signaling, cold stress

INTRODUCTION

Research in the last two decades has proved beyond doubt, the versatility of nitric oxide (NO) as an important signaling molecule in plants. It regulates numerous biological processes (Besson-Bard et al., 2008). Despite this, relatively little is known about its downstream signaling pathways. NO predominantly manifests its effects by post-translational modifications (PTMs) like S-nitrosylation, glutathionylation and tyrosine nitration. S-nitrosylation is the most investigated PTM, which regulates the physiological processes (Kovacs and Lindermayr, 2013). Once the physiological relevance of S-nitrosylation was established, the next phase of research focused on the identification of the putative S-nitrosylated targets, to establish the signaling mechanism.

S-nitrosylated proteins were identified from *Arabidopsis thaliana* (Lindermayr et al., 2005), *Kalanchoe pinnata* (Abat et al., 2008), *Brassica juncea* (Abat and Deswal, 2009), *Solanum tuberosum* (Kato et al., 2012), *Oryza sativa* (Lin et al., 2012) and *Pisum sativum* (Camejo et al., 2013). S-nitrosoproteome analysis is mostly done using NO donor because of the low concentration of endogenous S-nitrosothiols (SNOs). It is mandatory to identify and validate the endogenously S-nitrosylated proteins not only to confirm the targets identified using donors but also to understand their physiological relevance.

For the identification of S-nitrosylated proteins, biotin switch technique (BST, Jaffrey and Snyder, 2001) is used. It involves the selective reduction of the SNOs by ascorbate, their substitution with biotin and their purification by avidin-affinity chromatography. A major drawback of this procedure is the masking of the low abundant S-nitrosylated proteins by the abundant ones like Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, Abat and Deswal, 2009), RuBisCO activase (Tanou et al., 2012), glyceraldehydes-3-phosphate dehydrogenase (GAPDH, Maldonado-Alconada et al., 2010) and heat shock proteins (Maldonado-Alconada et al., 2010). These proteins saturate the avidin column and compete out the low abundant S-nitrosylated proteins. Besides hindering the detection of the low abundant targets, these also waste precious effort and time during MS identification. This prompted us to remove RuBisCO to improve the chances of getting the regulatory S-nitrosylated targets.

Recently, a NO-cold crosstalk was proposed at genes, lipid and protein level, but the regulatory mechanisms involved are still elusive (Sehrawat et al., 2013). Therefore, to get a better understanding of these signaling pathways, identification of the regulatory targets is essential. Previously, cold mediated inhibition of RuBisCO by S-nitrosylation was shown (Abat and Deswal,

2009), on the similar lines other signaling targets need to be functionaly validated. Therefore, the aim of this study was to demonstrate if the repertoire of cold responsive S-nitrosoproteome could be enriched by removing RuBisCO. Furthermore, the effect of S-nitrosylation on the superoxide dismtase (SOD) activity, a cold responsive S-nitrosylated target (identified in this study), was validated to understand its regulation by NO. In addition, to establish the NO signaling in cold, NO production and modulation of the *in vivo* thiol pool by NO was measured.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Brassica juncea var. pusa jaikisan seeds were obtained from The Indian Agricultural Research Institute, New Delhi, India. Seeds were surface sterilized with 70% ethanol for 10 min and soaked overnight in double distilled water. Seeds were placed in the wet germination paper rolls and kept overnight in dark. These were transfered to a growth chamber at 25°C under white fluorescent light (270 μmol/m^2/s, 16 h light/8 h dark) for 7 days.

COLD STRESS, SNP (SODIUM NITROPRUSSIDE) AND cPTIO (2-pHENYL-4,4,5,5-TETREMETHYL-IMIDAZOLINE-1-OXYL-3-OXIDE) TREATMENT

For cold stress, 7 days old seedlings were kept in a cold chamber at 4°C for 2–96 h under the same conditions as mentioned in the above section. Control seedlings were kept at 25°C. Seedlings were treated with SNP (a NO donor, 50, 100, 250 μM) or cPTIO (a NO scavenger, 100 μM). Following the treatment, the seedlings were rinsed with the double distilled water and blotted onto a filter paper and were immediately frozen in the liquid nitrogen.

NITRIC OXIDE MEASUREMENT

NO was measured using the NO measuring system (inNO, Innovative Instruments Inc.) following manufacturer's instructions. inNO consist of a nitric oxide meter, a sensor and a data acquisition system which measure free NO in the sample. NO measurement experiments were performed following (Modolo et al., 2005). In brief, seedlings (1:1, w/v) were homogenized in sodium phosphate buffer (100 mM, pH 7.4). Homogenate was centrifuged at 10,000 g (Beckman Coulter, Allegra 64R) for 10 min at 4°C. The supernatant was passed through two layer of cheese cloth and incubated for 1 h at 25°C with L-arginine (1 mM), NADPH (1 mM) with L-arginine (1 mM), NG-nitro-L-arginine methyl ester (1 mM, L-NAME), sodium nitrite (1 mM), NADH (1 mM) with sodium nitrite (1 mM) and sodium tungstate (1 mM) in different sets. NO was expressed as nM/ min /g FW.

THIOL POOL MEASUREMENT

The thiol pool was measured following (Ivanov and Kerchev, 2007) with some modifications like the control and cold (6 h) treated seedlings were homogenized in the extraction buffer in 1:1 (w/v) ratio. Additionally, the pellets, P1 and P2 were re-suspended

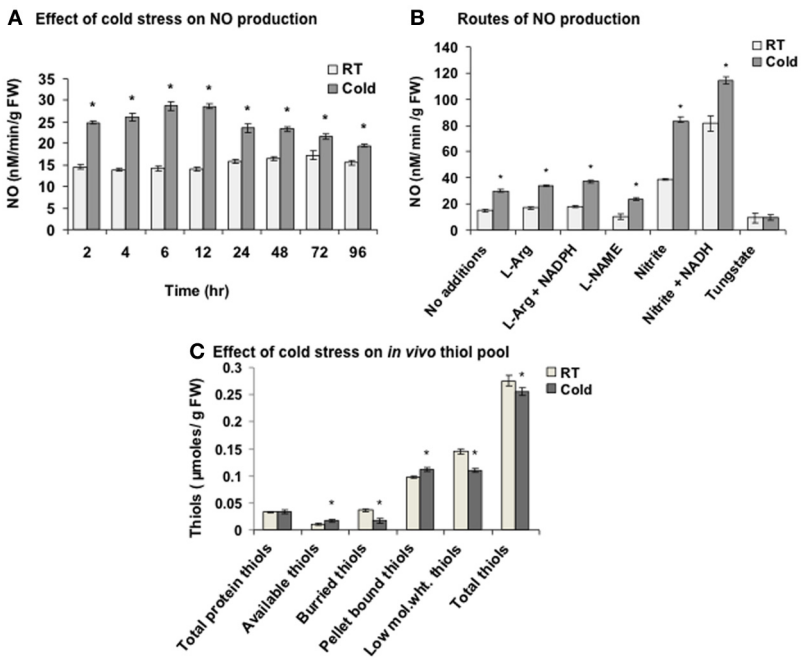

FIGURE 1 | Nitric oxide (NO) and *in vivo* thiol pool measurement in cold. (A) NO production measured using NO measuring system after the cold treatment (2–96 h) to the seedlings. **(B)** NO production in the extract from control (room temperature, RT) and cold (4°C) treated seedlings with L-arginine (L-Arg, 1 mM) alone or with NADPH (1 mM), L-NAME (1 mM), nitrite (1 mM) alone or with NADH (1 mM) and tungstate (1 mM). **(C)** Alterations in the thiol pool in cold (6 h) treated seedlings. Low mol. wt. thiols; low molecular weight thiols. Results represent mean ± SD from three independent experiments performed in triplicates. Asterisk (*) indicates significant differences between control and cold with $p = 0.05$ calculated using Student's *t*-test.

in a detergent solution using a sonicator (Ultrasonic Vibra cell), for the better solubilization of the thiols. A separate set of glassware was used to prevent any contamination with the metal ions. In addition, the entire experiment was performed at low temperature as the oxidation of thiols is temperature dependent. The results were expressed as μmoles -SH/g FW. Different fractions for analysis were: pellet bound thiols, pellet obtained after the first centrifugation (P1); available thiols, supernatant 1- supernatant 2 (S1–S2); low molecular weight thiols, S2; total protein thiols, pellet obtained after trichloroacetic acid (TCA) precipitation (P2); buried thiols, total protein thiols—available thiols; total thiols, total protein thiols + low molecular weight thiols + pellet bound thiols.

RuBisCO DEPLETION BY PEG PRECIPITATION AND IMMUNOAFFINITY PURIFICATION

For RuBisCO depletion, the seedlings were extracted (1:3, w/v) in 20 mM Tris-HCl (pH 7.0) containing 20% glycerol and 5 mM PMSF. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. Protein was estimated by Bradford assay (Bradford, 1976). The supernatant was used for the RuBisCO depletion experiments. For PEG precipitation, PEG 4000 [60% (w/v)] was added to the supernatant (5–15%) with stirring. After 30 min of stirring at 4°C, the extract was centrifuged at 16,000 g for 45 min. The pellet and the supernatant thus obtained were dissolved in the sample buffer and loaded on a 12% SDS-PAGE gel.

For the immunoaffinity purification, Seppro IgY RuBisCO Spin Column kit (Sigma–Aldrich) was used following the manufacturer's instructions. Briefly, the column was washed thrice before use with 500 μL tris buffered saline (TBS, 1 mM Tris-HCl, 150 mM NaCl, pH 7.4) to remove the suspension buffer. Immuno-capture of RuBisCO was performed by incubating the supernatant (90 μg protein) with the matrix for 15 min at 25°C with gentle shaking. After 15 min, the flow through was collected by centrifugation at 2000 rpm for 30 s. Unbound protein were removed by washing with TBS. Elution was done with the stripping buffer (100 mM glycine-HCl, pH 2.5) and the fractions were immediately neutralized with 1M Tris-HCl, pH 8.0.

DETECTION AND THE PURIFICATION OF THE S-NITROSYLATED PROTEINS

The S-nitrosylated proteins were detected and purified from RuBisCO depleted fractions by BST and neutravidin-agarose column chromatography following Abat and Deswal (2009) except that the GSNO, GSH, and DTT were removed using micro Bio-Spin 6 columns (Bio-Rad). For the purification of the cold modulated S-nitrosylated proteins, extraction and purification of RuBisCO depleted proteins was performed in dark to prevent the light induced degradation of SNOs. Separate Seppro columns were used for the control and the cold treated samples to avoid cross contamination. Stress induced S-nitrosylation was analyzed from the RuBisCO depleted fractions (5 mg) obtained from cold (6 h, 4°C) treated seedlings as mentioned above. S-nitrosylated proteins were resolved on 1-D and 2-D gels. Experiment was repeated with three biological replicates.

TWO-DIMENSIONAL ELECTROPHORESIS

Two dimensional electrophoresis was performed following Abat and Deswal (2009) with minor modifications. In the lysis buffer, 0.75% ampholytes was used to increase the solubilization of proteins. The gels were stained with the MS compatible silver staining as described by Yan et al. (2000). Three biological replicates were performed for each sample.

IMAGE ACQUISITION, DATA ANALYSIS AND PROTEIN IDENTIFICATION BY nLC-MS/MS

The gels were scanned using Alpha Imager (Alpha Innotech, Corporation). ImageMaster 2-D Platinum software (version 6.0, GE Healthcare, Sweden) was used for the spot detection in the 2-D gels. Protein spot pattern from the gels of three independent biological replicates were used to create a master gel in the first level match set. The gels were normalized in the percentage spot volume mode to reduce the differences in the protein loading and

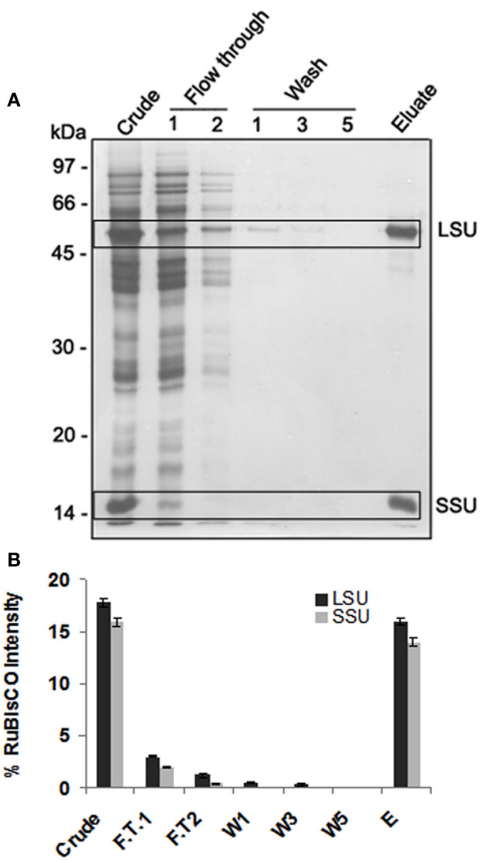

FIGURE 2 | 1-DE of RuBisCO depleted fractions and its comparison with the crude proteins displaying substantial removal of RuBisCO. (A) SDS-PAGE (12%) gel showing RuBisCO depletion after immunoaffinity purification using Seppro IgY-RuBisCO spin column kit. Large (LSU) and small (SSU) subunit of RuBisCO are marked in boxes. **(B)** Relative intensity of the polypeptides of LSU and SSU of RuBisCO quantified using densitometric scanning (AlphaImager software, Alpha Innotech Corporation). Polypeptide intensities were calculated by subtracting the background intensity. The results are representative of three biological replicates.

gel staining. This was followed by the formation of a second level match set where master gel of different samples was compared. Intensity of each spot is defined as the sum of the intensities of the pixels constituting that spot and is represented in the spot volume. Students's *t*-test ($p < 0.05$) was applied to determine any significant quantitative change.

For the MS identification, polypeptides/spots were manually excised from silver stained 1-D or 2-D gel. Identification was done at Proteomics International by Electrospray mass spectrometry on a 4000 Q TRAP mass spectrometer (Applied Biosystems). Utimate 3000 nanoflow LC system (Dionex, Bannockburn, IL, USA) was used for sample introduction as described in Bringans et al. (2008). The peak list obtained was submitted to the MASCOT search engine (http://www.matrixsciences.com) and was searched against the NCBInr (20130407 24070523 sequences; 8281664780 residues) in Viridiplantae. The search parameters were same as described in (Abat and Deswal, 2009) with peptide mass tolerance—±0.8 Da, and instrument type—ESI-QUAD-TOF. The significant hits identified by MASCOT probability analysis ($p < 0.05$) with mowse score 50 and above were selected. The unidentified/hypothetical proteins were subjected to BLASTP search against the NCBInr protein database to assign function to the unnamed or unknown proteins.

SUPEROXIDE DISMUTASE AND FRUCTOSE BISPHOSPHATE ALDOLASE ACTIVITY ASSAY

For the enzyme assays, the seedlings were extracted in the HEN buffer (250 mM Hepes-NaOH pH 7.7, 1 mM EDTA, 0.1 mM Neocuproine, pH 7.4, 1:3, w/v) and the homogenate was centrifuged at 14,000 g for 25 min at 4°C. The supernatant was passed through two layers of the cheese cloth and was incubated without or with GSNO (100-500 μM) or GSH (250 μM) in the dark for 20 min at 25°C. For the DTT treatment, after incubation with GSNO (100 μM), the samples were incubated with DTT (10 mM) in dark for 40 min. GSNO, GSH and DTT were removed using Micro Bio-Spin 6 columns (Bio-Rad).

The total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium [NBT, (Beyer and Fridovich, 1987)]. The reaction mixture (1.5 ml) contained 33 μg of protein extract, phosphate buffer (50 mM, pH 7.8), EDTA (0.1 μM), methionine (13 mM), NBT (75 μM) and riboflavin (2 μM). One unit of SOD activity is

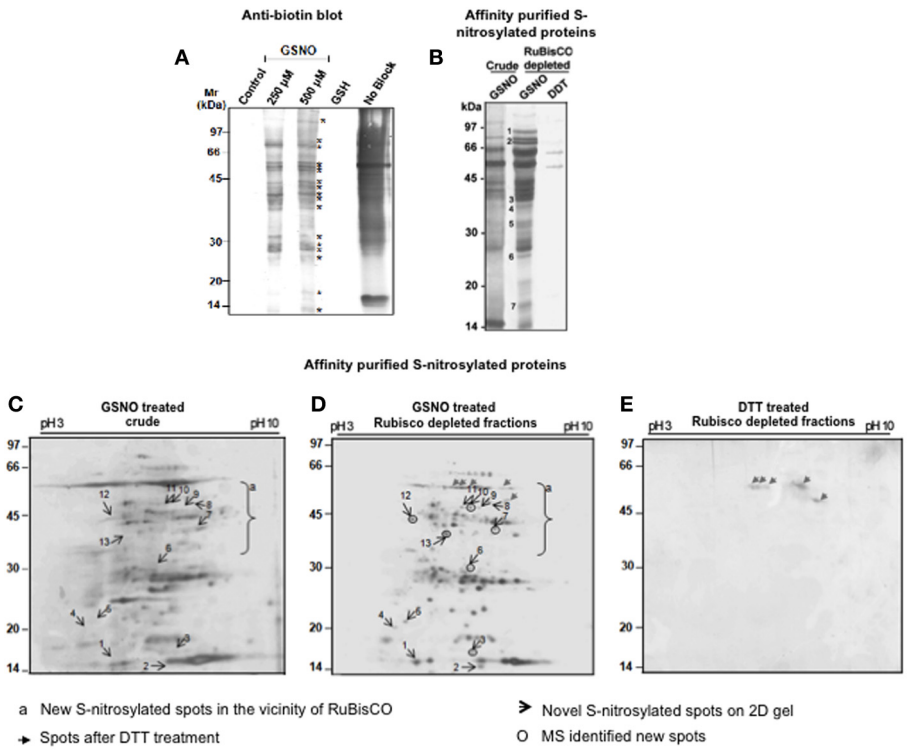

FIGURE 3 | Detection and purification of the S-nitrosylated proteins from the RuBisCO depleted fractions. (A) RuBisCO depleted extracts containing 250 μg protein were treated with or without 250 μM and 500 μM GSNO or GSH (250 μM) and labeled with biotin using biotin switch technique. Additionally, proteins without MMTS (no block) treatment served as a control for the blocking step. Proteins were resolved on a 12% SDS-PAGE gel and blotted onto nitrocellulose membrane. Biotinylated proteins are marked with asterisk (*). **(B)** For the purification of the S-nitrosylated proteins, proteins (5 mg) treated with GSNO (500 μM) were subjected to biotin switch method, followed by their purification using neutravidin affinity chromatography. As a negative control, proteins were first S-nitrosylated with GSNO (500 μM) and then reduced with 10 mM DTT. Eluates were separated on a 12% SDS-PAGE gel. Purified S-nitrosylated polypeptides absent in the crude are marked with numbers. **(C)** and **(D)** 2-D gels (12%) of purified S-nitrosylated proteins from the crude and RuBisCO depleted fractions and after GSNO (500 μM) treatment. **(E)** 2-D gel (12%) of the GSNO (500 μM) and DTT (10 mM) treated RuBisCO depleted fractions. Gels were silver stained by MS compatible silver staining and analyzed using ImageMaster 2-D Platinum software.

defined as the amount of enzyme which causes 50% inhibition in the NBT reduction. Optical density was recorded at 560 nm using a UV-spectrophotometer (Beckman Coulter, DU-730).

Fructose bisphosphate aldolase activity assay was done based on Boyer's modification of hydrazine assay following Richards and Rutter (1961). This assay is based on reaction of 3-phosphoglyceraldehyde (product of fructose bisphosphate aldolase) with hydrazine to form hydrazone which absorbs at 240 nm. One unit of enzyme is defined as a change in absorbance/min at 25°C. The assay mixture contained 0.012 M fructose-1, 6 bisphosphate, 0.1 mM EDTA containing 3.5 mM hydrazine sulfate. After recording the absorbance 240 nm for 10 min, the enzyme (25 µl) was added and the absorbance was recorded further for 10 min.

STATISTICAL ANALYSIS

Intensity of polypeptide in SDS-PAGE gels was quantified by densitometric scanning (AlphaImager software, Alpha Innotech Corporation) with three repeats. The data shown in the NO measurement, thiol pool analysis and the enzymatic assay represents mean ± SD from three independent experiments performed in triplicates and significant differences were calculated by Student's *t*-test with $p \leq 0.05$.

RESULTS

COLD STRESS ENHANCED ENDOGENOUS NITRIC OXIDE PRODUCTION AND MODIFIED THE *in vivo* THIOLS

NO content was measured in the cold (4°C) treated seedlings using NO measuring system. The sensing element of the iNO sensor has a NO selective permeable membrane. Cold stress led to NO evolution right from 2 h with maximum NO accumulation (2 fold) at 6 h (**Figure 1A**). In control (25°C, RT), negligible increase (at 72 h) was observed. Nitric oxide synthase (NOS)-like enzyme and nitrate reductase (NR) are the two key enzymes responsible for the NO production in plants. Addition of L-arginine (1 mM, substrate of NOS-like enzyme) alone or with NADPH (1 mM, cofactor of NOS) showed 1.1 and 1.23 fold increase respectively in NO in cold (6 h), while L-NAME (1 mM, an inhibitor of NOS) brought it back to the basal level (**Figure 1B**). In contrast, nitrite (1 mM, a substrate for NR) alone or along with NADH (1 mM, a cofactor of NR) increased the NO production by 2.78 and 3.72 fold respectively indicating primarily NR mediated NO production in cold. Higher NO production than the *in vivo* NO generating capacity of the plant, could be due to the higher concentrations of the substrate and cofactors being provided from the outside. A decrease in NO to the basal level by tungstate (1 mM, an inhibitor of NR) in cold confirmed the results. The control (RT) sets showed a similar trend.

As NO modulates the cellular thiols, these were quantified in cold stress. Thiols are broadly categorized into protein-based (high molecular weight), non-protein based (low molecular weight) and pellet bound thiols. Protein-based thiols are further categorized as available and buried thiols. Low molecular weight thiols include GSH and free cysteines. Pellet bound thiols are the thiols present in the broken organelles and cell membranes. Cold stress increased available thiols and pellet bound thiols by 54.5% and 14.2% respectively, while decreased the buried thiols and low molecular weight thiols by 53.8% and 24.1% respectively (**Figure 1C**). Overall, 7.2% decrease in the total thiols was observed in cold. One of the reason for this decrease could be the reaction of cold induced NO with low molecular weight thiols like GSH to yield GSNO leading to S-nitrosylation.

S-nitrosylation analysis of the regulatory targets is challenging due to their low abundance and masking by the abundant proteins like RuBisCO. Therefore, to increase the proteome coverage, RuBisCO was removed, There are reports of successful RuBisCO removal by PEG precipitation (Xi et al., 2006), affinity purification (Cellar et al., 2008), higher DTT concentrations (Cho et al., 2008), Ca^{2+}/phytate fractionation (Krishnan and Natarajan, 2009) and protamine sulfate precipitation (Kim et al., 2013). Here, PEG precipitation and RuBisCO IgY affinity chromatography were used for RuBisCO removal and S-nitrosylation analysis.

IMMUNOAFFINITY REMOVAL OF RuBisCO AND MS IDENTIFICATION OF THE AFFINITY PURIFIED S-NITROSYLATED PROTEINS FROM THE RuBisCO DEPLETED FRACTIONS

PEG precipitation was not effective as along with RuBisCO other proteins were also depleted (data not shown). Seppro RuBisCO spin columns (IgY affinity purification) removed 83% and 87.5% of large and small subunit of RuBisCO respectively as shown

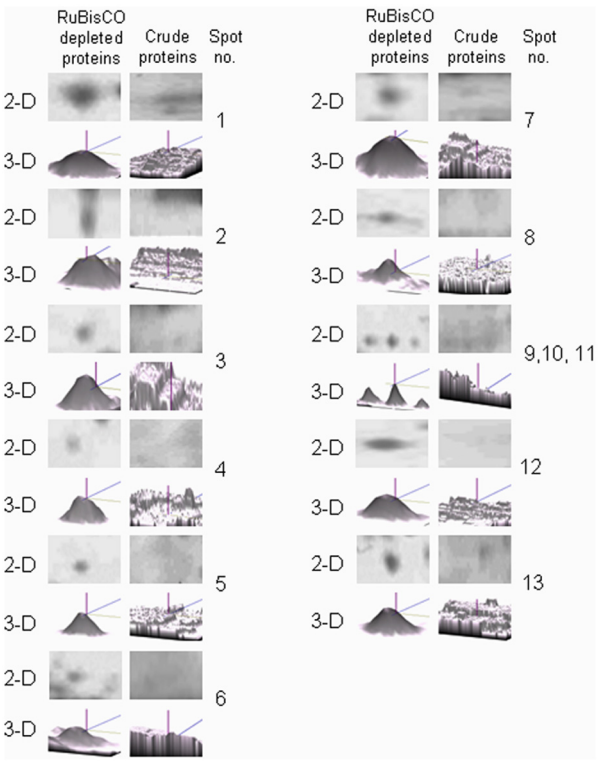

FIGURE 4 | Three-dimensional (3-D) view of unique protein spots in the RuBisCO depleted fractions generated using ImageMaster 2-D Platinum software (GE Healthcare).

Table 1 | S-nitrosylated proteins identified from NO donor (GSNO) treated RuBisCO depleted fractions of *B. juncea*.

Protein name	Acc. no.	M.S.	S.C.(%)	Matched peptides [Identified peptide (ion score)]	MW(Da)/pi		Spot number	Reported as S-nitrosylated in crude *B.juncea*	Reported as S-nitrosylated in any other study	Functional category
					Observed	Theoretical				
Thioredoxin H-type (*Brassica rapa*)	gi|11135129	106	8%	1[VDVDELATVAK (106)]	15000/7.3	13749/5.3	03	No	Maldonado-Alconada et al., 2010; Kato et al., 2012; Lin et al., 2012	Stress/ signaling/ redox
Predicted protein, (Salt responsive protein 2, *Hordeum vulgare*)	gi|326514010 (NP_001234 228)	51	5%	2[LATGEPLR (36)]	29870/7.0	51137/8.7	06	No	–	
Hypothetical protein ARALYDRAFT_486711 (Peptidyl-prolyl cis-trans isomerase CYP20-3, *Arabidopsis lyrata*)	gi|297817542 (NP_191762)	113	11%	3[IVMGLFGEVVPK(52)]	43200/8.3	26725/8.6	07	No	Lindermayr et al., 2005; Tanou et al., 2012	
Myrosinase (*Brassica napus*)	gi|127733	102	3%	2[GYAIGTDAPGR (79)]	51480/6.8	63266/6.6	10	Yes	–	
Malate dehydrogenase, mitochondrial precursor (*Brassica napus*)	gi|2497857	240	15%	5[SQVVGYMGDDNLAK (71)]	42510/4.7	35860/8.8	12	No	Lindermayr et al., 2005; Tanou et al., 2012	Metabolism
Fructose-bisphosphate aldolase, class I (*Arabidopsis thaliana*)	gi|18420348	457	15%	6 [LDSIGLENTEANR(95)]	38054/5.9	43029/6.7	13	Yes	Lindermayr et al., 2005; Maldonado-Alconada et al., 2010; Tanou et al., 2012	

*Protein identification was performed by nLC-MS/MS using Mascot search engine using NCBI database. Spots were identified from **Figure 3D**. Proteins in bold indicate novel S-nitrosylation targets which are not identified in any plant system. The best matching peptide identifying the protein with ion score is reported, M.S., Mowse Score; Acc. no., Accession number; Seq. Cov., Sequence coverage.*

Table 2 | Cold responsive S-nitrosylated proteins from RuBisCO depleted fractions of cold treated *B. juncea* seedlings.

Protein name	Acc. no.	M.S.	S.C.(%)	Matched peptides [Identified peptide (ion score)]	MW(Da)/pI		Spot/ polypeptide number and patter	Reported as S-nitrosylated in crude	Reported as S-nitrosylated protein in anyother study	Functional category
					Observed	Theoretical				
Putative lactoylglutathionelyase (*Brassica rapa*)	gi|157890952	487	36%	10[IANQELGGKITR (84)]	15657/6.2	32006/5.3	2, U	No	–	Stress/ signaling/redox
Daikon cysteine protease RD21 (*Raphanus sativus*)	gi|219687002	246	16%	5[NGGIDTEEDYPYK (105)]	22055/5.5	32085/4.27	4, U	No	Maldonado-Alconada et al., 2010	
Vacuolar calcium binding protein (*Raphanus sativus*)	gi|9049359	120	9%	2[TEETPAVVEEEK (61)]	29003/6.6	27094/4.1	8, D	No	–	
Fe-superoxide dismutase, partial (*Arabidopsis thaliana*)	gi|166700	50	6%	2[RPDYIK (28)]	41087/6.7	25409/6.3	11, U	Yes	Lin et al., 2012; Tanou et al., 2012	
Glutathione S-transferase (Brassica napus)	gi|87294807	160	17%	4[SPLLLQSNPIHK(52)]	95510	24887	*3, U*	Yes	Tanou et al., 2009	
Chaperonin 10 (*Arabidopsis thaliana*)	gi|3057150	77	8%	3[YAGTEVEFNDVK (49)]	95510	26912	*3, U*	No	Lindermayr et al., 2005	
Epithiospecifier protein (Brassica rapa)	gi|211905345	428	33%	16[FITKLDEEGGPEAR(74)]	53640	37890	*6, U*	No	–	
MLP-like protein 328 (*Arabidopsis thaliana*)	gi|18379240	108	17%	7[GLEGHVMEQLK(47)]	40430	17616	*8, U*	Yes	Lindermayr et al., 2005	
Soluble inorganic pyrophosphatase 1 (*Arabidopsis thaliana*)	gi|15242465	222	11%	3[MEVATDEDFTPIK(90)]	29010/7	33644/5.7	7, D	No	–	Metabolism
Putative fructose-bisphosphate aldolase (*Arabidopsis thaliana*)	gi|14539316	176	11%	3[LDSIGLENTEANR(94)]	43041/6.9	37291/8.8	12, U	Yes	Lindermayr et al., 2005; Maldonado-Alconada et al., 2010; Tanou et al., 2012	
Fructose-bisphosphate aldolase, class I (*Arabidopsis thaliana*)	gi|18420348	713	19%	I5[LDSIGLENTEANR(116)]	52132/7	43029/6.7	14, U	Yes	Lindermayr et al., 2005	

(Continued)

Table 2 | Continued

Protein name	Acc. no.	M.S.	S.C.(%)	Matched peptides [Identified peptide (ion score)]	MW(Da)/pI		Spot/ polypeptide number and patter	Reported as S-nitrosylated in crude	Reported as S-nitrosylated protein in anyother study	Functional category
					Observed	Theoretical				
AT4g38970/F19H22_70 (Fructose biphosphate aldolase 1, Arabidopsis thaliana)	gi|16226653	334	32%	I8[LDSIGLENTEANR(47)]	53640	38858	*6, U*	Yes	Lindermayr et al., 2005	
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic (Sinapinis alba)	gi|120675	599	59%	25[VPTVDVSWDLTVR(75)]	53640	37015	*6, U*	No	Lindermayr et al., 2005; Maldonado-Alconada et al., 2010	
Sedoheptulose-bisphosphatase *(Arabidopsislyrata)*	gi|297816906	675	27%	18[LTGVTGGDQVAAAMG IYGPR (134)]	68081/6.2	42861/6.0	15, U	Yes	Tanou et al., 2009	Photosynthesis
Sedoheptulose-bisphosphatase (Arabidopsislyrata)	gi|297816906	358	22%	11[GIFTNVTSPTAK(70)]	42250	42861	*7, U*	Yes	Tanou et al., 2009	
Beta-carbonic anhydrase, chloroplastic (Brassica napus)	gi|297787439	374	29%	11[VISELGDSAFEDQ CGR(82)]	95510	36127	*3, U*	No	Lindermayr et al., 2005; Tanou et al., 2012	
Oxygen-evolving enhancer protein 2, chloroplastic (Pisum sativum)	gi|131390	169	10%	7[FVEDTASSFSVA(76)]	37500	28201	*9, U*	No	Lindermayr et al., 2005	
Hypothetical protein SORBIDRAFT_02g002690, (23 kDa polypeptide of PS II)	gi|242047384 (AAB82135)	122	8%	3[HQLITATVSDGK(63)]	37500	27718	*9, U*	No	Tanou et al., 2012	
Unknown protein 18 *(Vitis rotundifolia)*	gi|205830697	149	100%	3[TNAENEFVTIKK((78)]	29950/5.3	1393/5.85	9. D	No	–	Unknown
Unknown protein 18 *(Vitis rotundifolia)*	gi|205830697	158	100%	1[TNAENEFVTIK(80)]	22076/5.8	1393/5.85	5, D	No	–	
Unnamed protein product (Thellungiella halophila)	gi|312282755	500	53	2[(VPTVDVSWDLTVR(75)]	53640	32088	*6,U*	No	–	

*Protein identification was performed by nLC-MS/MS using Mascot search engine using NCBI database. Spots/polypeptides were identified from **Figure 5B** and Supplementary material S1. Proteins in bold indicate novel S-nitrosylation targets which are not identified in any plant system. Proteins in italics indicate the protein identified from 1-D gel. The best matching peptide identifying the protein with ion score is reported, M.S., Mowse Score; Acc. no., Accession number; Seq. Cov., Sequence coverage.*

by the densitometric quantification (**Figures 2A,B**). For the S-nitrosylation analysis, RuBisCO depleted fraction, F.T.1 (flow through 1) was used.

S-nitrosylated proteins were detected in the RuBisCO depleted fractions by BST. The RuBisCO depleted fractions (0.8 μg/ul) were dissolved in the HEN buffer and GSNO was used for mimicking the *in vivo* S-nitrosylation. Immunoblot of GSNO (250 and 500 μM) treated fractions showed 17 immunopositive polypeptides (**Figure 3A**, marked with *) which were absent in the control and GSH treated fractions (250 μM, an inactive analog of GSNO), suggesting specific S-nitrosylation. Omission of the blocking (positive control), showed non-specific biotinylation of the free thiols indicating good efficacy of the procedure.

Affinity purified S-nitrosylated proteins showed 16 polypeptides on a 12% gel (**Figure 3B**), including 7 polypeptides (**Figure 3B**, marked with numericals) which were absent in the crude, showing that these were competed out by RuBisCO. The DTT-treated fraction showed only two polypeptides (52 and 60 kDa) indicating reversibility of the reaction.

RuBisCO depleted purified S-nitrosylated proteins resolved as 110 spots, while crude S-nitrosylated proteins showed 97 spots on the 2-D gel (**Figures 3C,D**). DTT treatment (a negative control) showed five spots (**Figure 3E**, marked with gray arrows), molecular weight of which corresponded with the DTT treated polypeptides (**Figure 3B**), showing repeatability of the results. A spot-to-spot comparison and statistical analysis using the ImageMaster 2-D Platinum software, detected 13 new spots in the RuBisCO depleted fractions with a significant ($p < 0.05$) change in the abundance (**Figures 3C,D**). A three-dimensional view of these spots confirmed their increased abundance (**Figure 4**). Interestingly, seven (spot 7, 8, 9, 10, 11, 12, and 13), of these spots were in the vicinity of RuBisCO as seen on the 2-D gel of crude and RuBisCO depleted S-nitrosylated proteins (**Figures 3C,D**, marked with a), showing that RuBisCO masked these spots in the crude. These spots were identified using nLC-MS/MS as peptidyl-prolyl cis-trans isomerase (PPIase), malate dehydrogenase and fructose-bisphosphate aldolase (**Table 1**).

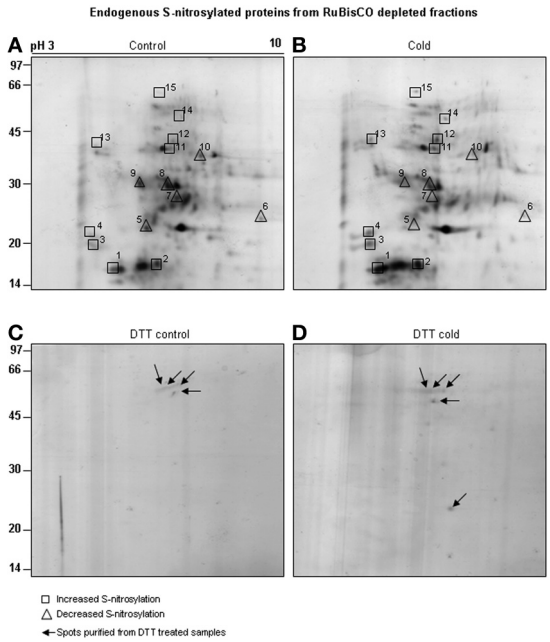

FIGURE 5 | Purification of cold responsive S-nitrosylated proteins. RuBisCO depleted proteins (5 mg) from control **(A)** and cold **(B)** treated seedlings were subjected to BST and neutravidin affinity purification. Purified S-nitrosylated proteins were resolved on 2-D gel using non-linear IPG strips (13 cm, pH 3-10) and 12% SDS-PAGE. As a control, RuBisCO depleted proteins from the control **(C)** and cold **(D)** seedlings were treated with DTT (10 mM) and the purified spots were resolved on 12% SDS-PAGE (marked with arrows). Gels were stained using MS compatible silver staining and analyzed using ImageMaster 2-D Platinum software. Spots showing increased S-nitrosylation intensity after cold stress are marked with boxes and decreased S-nitrosylation by triangle.

COLD RESPONSIVE PROTEINS FROM THE RuBisCO DEPLETED FRACTIONS SHOWED STRESS/SIGNALING/REDOX RELATED FUNCTION AS A MAJOR CATEGORY OF THE S-NITROSYLATED PROTEINS

We have earlier shown that cold stress modulated S-nitrosylation and few targets were identified (Abat and Deswal, 2009). To enrich the repertoire, cold responsive S-nitrosylated proteins were purified from the RuBisCO depleted fractions of the cold treated seedlings. Cold stress of 6 h was chosen as it showed maximum NO and SNO (Abat and Deswal, 2009) production. Eleven endogenously S-nitrosylated polypeptides (24-108 kDa) were resolved on the SDS-PAGE gel (Supplementary material S1, marked with numericals). These were identified as 11 proteins with a significant score (**Table 2**). DTT treated cold samples showed, only three faint polypeptides (Supplementary material S1, marked with *).

Neutravidin-affinity purified cold responsive S-nitrosylated proteins resolved as 78 spots, out of which 15 spots showed differential S-nitrosylation (**Figures 5A,B**). Of these, 9 spots showed increased (**Figures 5A,B**, marked with square), while 6 spots showed decreased (**Figures 5A,B**, marked with triangle) S-nitrosylation. DTT treated sample showed four (**Figure 5C**) and five (**Figure 5D**) spots in the RuBisCO depleted fractions from control and cold treated seedlings. The 2-D gel showed better resolution of the low molecular weight S-nitrosylated proteins than the 1-D gels. Most abundant spots (10) showing differential S-nitrosylation (**Figure 5B**) were identified by nLC-MS/MS (**Table 2**). The difference in the theoretical and the experimental molecular weights of some of the identified proteins could be due to different isoforms, PTMs or degradation of the proteins.

Overall, the functional categorization of the cold responsive S-nitrosylated targets showed stress/signaling/redox related functions to be the largest functional category. The second largest category was of metabolic proteins. The third category included photosynthetic targets, while unknown targets were least in number. It is worth mentioning that putative lactoylglutathione lyase/glyoxylase I (Gly I), epithiospecifier protein, vacuolar calcium binding protein, inorganic pyrophosphatase I, unnamed protein products and unknown proteins are identified as S-nitrosylated proteins for the first time in plants.

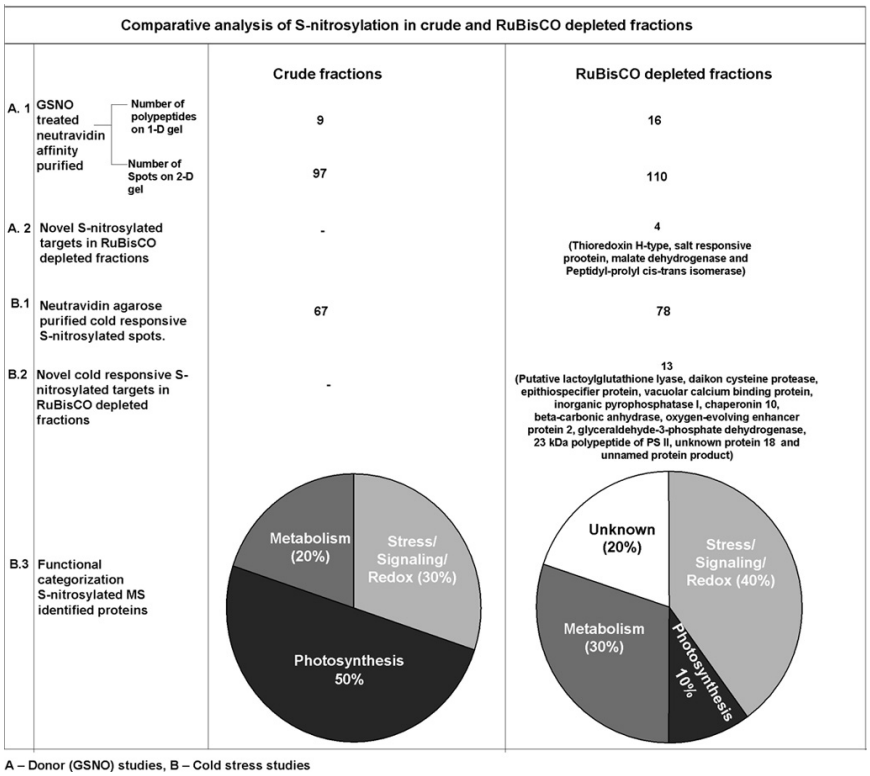

FIGURE 6 | Comparison of S-nitrosylation in crude and RuBisCO depleted fractions showing increased S-nitrosoproteome coverage by RuBisCO depletion.

A comparison of S-nitrosylation of the crude with RuBisCO depleted fractions showed that RuBisCO depletion increased polypeptide/spot number on the 1-D/2-D gels, indicating its effectiveness in S-nitrosylation analysis (**Figure 6**). MS identification further supported the results, as novel targets were identified in the GSNO and cold treated RuBisCO depleted fractions. Moreover, the functional categorization of the cold responsive S-nitrosylated proteins showed a shift from the photosynthetic targets to the redox/stress/signaling and metabolic proteins in the RuBisCO depleted fractions in comparison with the crude. This suggests a functional switching over from the normal physiology to signaling for combating the stress. Interestingly, a new category of unknown proteins was also observed in RuBisCO depleted fractions. Therefore, RuBisCO depletion seems to be a fruitful strategy in unraveling the physiological functions of S-nitrosylation and in enhancing the S-nitrosoproteome coverage.

EFFECT OF NITRIC OXIDE AND COLD STRESS ON SUPEROXIDE DISMUTASE ACTIVITY

In the present study, Fe-SOD showed an increase in S-nitrosylation in cold (spot number 11, **Figures 5A,B** and **Table 2**). To know, the effect of S-nitrosylation on the SOD activity, the extracts were incubated with GSNO (100 μM), which showed 49% increase in the activity (**Figure 7A**). Cold treatment showed 50% increase in the SOD activity. DTT (10 mM) brought down the activity to 27 and 33% in the GSNO and cold treated samples respectively. As DTT treatment did not show 100% reversal, this indicated the role of other NO based PTMs, besides S-nitrosylation in regulating SOD. To further confirm these results, NO donor (SNP) and inhibitor (cPTIO) treatment was given to control (RT) and cold (4°C) treated seedlings and the extract was used for the activity assay. SNP (50 μM) increased the activity to 84.1% in cold, while it was not promoting the activity at 100 and 250 μM (**Figure 7B**). Control showed a similar trend. cPTIO reduced the increased activity to the basal level. These results showed cold stress mediated SOD activation by S-nitrosylation.

DISCUSSION

Recently, the role of NO as a key component in cold stress signaling was emphasized (Liu et al., 2010; Gupta et al., 2011; Bai et al., 2012; Wang et al., 2012; Sehrawat et al., 2013). In the present report, evidences for the NO signaling in cold stress are provided. Endogenous NO increased by 2 fold after 6 h of cold stress and NR seems to be a major contributor in the NO production. NR dependent NO production in cold was earlier shown in *A. thaliana* leaves (Cantrel et al., 2011) and *Baccaurea ramiflora* seeds (Bai et al., 2012). NOS-like enzyme dependent NO production in cold stress is reported in *Pisum sativum* leaves (Corpas et al., 2008), *Chorispora bungeana* suspension cultures (Liu et al., 2010), *Solanum lycopersicum* fruits (Zhao et al., 2011), *B. juncea* seedlings (Talwar et al., 2012) and *Camellia sinensis* pollen tubes (Wang et al., 2012). The enzymes involved in cold

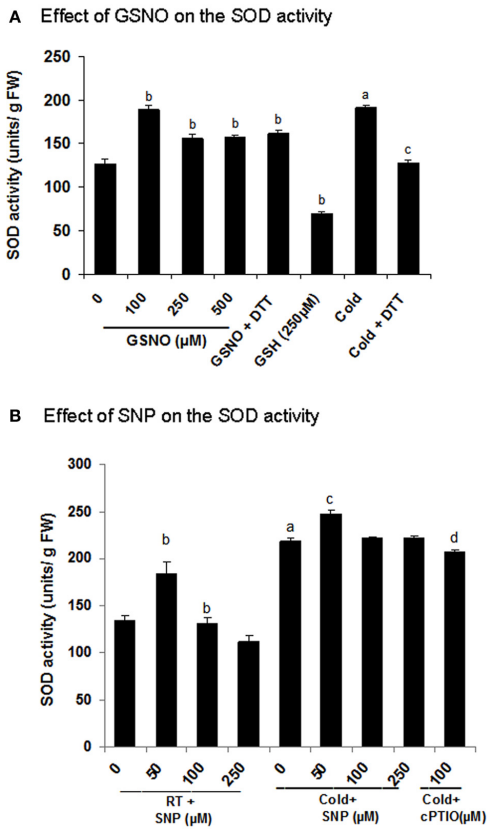

FIGURE 7 | Functional validation of superoxide dimutase (SOD) as a cold responsive S-nitrosylated protein. (A,B) Effect of GSNO and SNP on the SOD activity measured using NBT reduction assay. For the *in vitro* assays, extracts were incubated with or without GSNO (100 μM, 250 μM and 500 μM) or GSH (250 μM) prior to the activity analysis. Incubation with DTT (10 mM) was also done after GSNO (100 μM) treatment to check the reversal. Seedlings were treated with SNP (50–250 μM) and cPTIO (100 μM) with or without cold stress. Extracts from these samples were used for the assay. Error bars represents standard deviation from three independent experiments (biological repeats) performed in triplicates (technical repeats). Statistical significance was determined by Student's *t*-test. In **(A)** statistically significant difference ($p < 0.05$) between RT (control) and cold is shown (by a), control and GSNO (by b) and cold and DTT (by c). In **(B)** values with the same alphabets are showing significant difference ($p < 0.05$) between RT and cold (by a), control and SNP (by b), cold and SNP (by c) and cold and cPTIO (by d).

induced NO production vary with plant system, tissue type and stress, indicating differential regulatory mechanism(s) of NO production.

Cold stress alters the cellular redox homeostasis, while thiols play a significant role in its maintenance. In this study, an increase in the available thiol groups in cold was observed, indicating a shift from the buried to the available thiols, probably due to conformational change in the proteins. Interestingly, protein based thiols constituted 57% of the cold modulated total thiols, while low molecular weight thiols constituted 43%, indicating that both are contributing almost equally in maintaining the redox homeostasis. Unlike protein based thiols, low molecular weight thiols showed a decrease after cold as observed in heat treated pea seedlings (Ivanov and Kerchev, 2007) and cadmium treated *Salsola kali* leaves (Rosa et al., 2005). This decrease could be due to the utilization of low molecular weight thiols in S-nitrosylation of proteins. To establish this, S-nitrosylation was analyzed.

The major hurdle in the S-nitrosylation analysis in cold stress treated seedlings was RuBisCO, the most abundant S-nitrosylated protein. It competes with other S-nitrosylated proteins and hinders their resolution and MS identification. Therefore, RuBisCO (more than 80%) was removed from *B. juncea* crude extracts using immunoaffinity purification as it is quite conserved across the plant species.

To test, if RuBisCO removal improves efficacy of S-nitrosylation analysis, BST of RuBisCO depleted fractions (using GSNO) was performed. It improved the protein resolution as 7 new polypeptides (on the 1-D gel) and 13 new spots (on the 2-D gel) were observed. Increased polypeptide/spot number also suggests improved efficacy of the BST and neutravidin affinity chromatography. Moreover, it also enhanced the identification of the regulatory targets (thioredoxin, salt-responsive protein, PPIases and malate dehydrogenase), which earlier escaped detection in the crude (Abat and Deswal, 2009).

Cold stress increased the S-nitrosylation of Gly I, cysteine protease, Fe-SOD and fructose biphosphate aldolase, while decreased the S-nitrosylation of vacuolar calcium binding proteins, inorganic pyrophosphatase and unknown proteins (**Table 2**). Overall, the S-nitrosoproteome coverage of cold stress responsive signaling and redox related targets was increased by RuBisCO depletion.

In the present study, it is shown that cold induced NO causes increased S-nitrosylation of SOD and contributes to superoxide dismutation and ROS detoxification. S-nitrosylation of Fe-SOD was also shown in the salinity treated citrus leaves (Tanou et al., 2012), while Cu/Zn SOD was identified as a S-nitrosylated target in Arabidopsis (Lindermayr et al., 2005) and rice (Lin et al., 2012). This data is consistent with the previous report where increased S-nitrosylation of the enzymes of ascorbate glutathione cycle [ascorbate peroxidase, glutathione reductase and dehydroascorbate reductase (DHAR)] reduced desiccation-induced ROS accumulation and eventually enhanced the desiccation tolerance in *Antiaris toxicaria* seeds (Bai et al., 2011). Besides SOD, thioredoxin (H-type) is also S-nitrosylated and acts as a redox regulator of the transcription factors including non-expressor of pathogenesis related protein (NPR1, Tada et al., 2008), which further regulates the expression of the defense responsive genes. Overexpression of thioredoxin (H-type) in transgenic rice, induced the expression of chaperones in seeds (Wakasaa et al., 2013).

The identified proteins also include novel S-nitrosylated targets like Gly I, a vacuolar calcium binding protein (CaB) and inorganic pyrophosphatase 1. Although, tyrosine nitration of Gly I in salt stress was shown in citrus (Tanou et al., 2012), this is the first report of S-nitrosylation of Gly I in plants. The identified CaB (involved in maintaining calcium homeostasis) showed similarity with a unique CaB from *Raphnus sativus* (Yuasa and Maeshima, 2000). S-nitrosylation of CaB, suggest a cross-talk between NO and calcium signaling. To the best of our knowledge, till date the

role of this unique CaB is not investigated in stress, therefore it would be interesting to analyze its role in calcium signaling in cold. Overexpression of Arabidopsis inorganic pyrophosphatase in *E. coli*, conferred enhanced tolerance to abiotic stress (Yoon et al., 2013).

Identification of myrosinase and epithiospecifier protein (a novel S-nitrosylated protein), involved in the glucosinolates hydrolysis as targets, suggest the role of S-nitrosylation in regulating "glucosinolate hydrolysis pathway." This pathway is specific to Brassicaceae and is involved in protection against abiotic stress (Martinez-Ballesta et al., 2013).

Three enzymes of Calvin cycle namely fructose biphosphate aldolase, sedoheptulose-1,7-bisphosphatase and GAPDH were identified as cold responsive S-nitrosylated targets. Fructose biphosphate aldolase, is a cold responsive protein (Hashimoto and Komatsu, 2007). In the present study, an increase in the S-nitrosylation in cold was observed (spot number 12 and 14, **Figures 5A,B** and **Table 2**). Fructose biphosphate aldolase activity with GSNO (a NO donor), showed a dose dependent increase, while treatment with GSH (an inactive analog of GSNO) had no effect (Supplementary material S2). DTT (a reductant) reduced the activity back to the control level. These results showed a positive regulation of fructose biphosphate aldolase by S-nitrosylation. Increased activity of sedoheptulose-1,7-bisphosphatase enhanced salt stress tolerance in transgenic rice seedlings (Feng et al., 2007). Re-localization of GAPC1 (an isoform of cytosolic GAPDH) to the nucleus in cadmium treated *A. thaliana* seedlings, indicated its role in stress signaling (Vescovi et al., 2013).

Interestingly, after the RuBisCO removal six hypothetical/uncharacterized/unnamed proteins were identified. These were searched in NCBInr protein database using BLASTP. Hypothetical protein ARALYDRAFT_486711, hypothetical protein SORBIDRAFT_02g002690 and predicted protein were identified as PPIase, a 23 kDa polypeptide of PS II and a salt responsive protein 2 respectively. However, unknown protein 18 and unnamed protein product could not be identified, probably these are not yet reported. PPIases identified in this study are ubiquitous proteins, mediating protein folding in cold stress (Budiman et al., 2011). Additionally, ROC4 (only cyclophilin in the stroma of the chloroplast) is shown to have PPIases activity and is involved in the repair of photo-damaged PSII in *A. thaliana* (Cai et al., 2008). Chaperonin besides modulating protein folding, also regulates Fe-SOD activity (Kuo et al., 2013).

Most of the validated targets for S-nitrosylation are negatively regulated by S-nitrosylation [as reviewed by Astier et al. (2012)]. In contrast, there are very few targets like TGA1 (Lindermayr et al., 2010), ascorbate peroxidase (Bai et al., 2011), glutathione

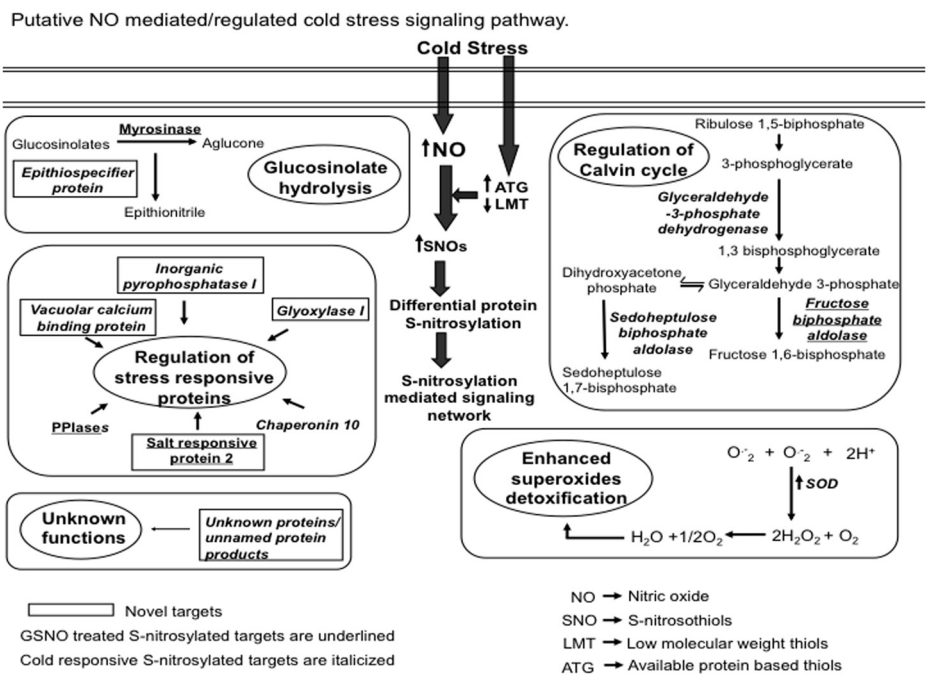

FIGURE 8 | A proposed model showing the S-nitrosylation mediated cold stress signaling. Cold stress increased nitric oxide (NO) production. This increased NO reacts with low molecular weight thiols (LMT) such as glutathione to produce S-nitrosoglutathione (GSNO). Available thiol groups (ATGs) also showed an increase in response to the cold stress. GSNO reacts with these ATGs to produce S-nitrosothiols (SNOs). Increased SNOs promote the S-nitrosylation of constitutive as well as the regulatory proteins. S-nitrosylation of superoxide dismutase (SOD) reduces the cellular damage caused by reactive oxygen species by scavenging superoxide radicals (O_2^-). S-nitrosylation of myrosinase and epithiospecifier protein suggests the probable role of NO in regulating glucosinolates hydrolysis pathway. Identification of vacuolar calcium binding protein, glyoxylase I, peptidyl-prolyl cis-trans isomerase (PPIases) and chaperonin 10 could be associated with the regulation of stress responses. The proposed model also reflects the physiological relevance of S-nitrosylation in regulating the Calvin cycle.

reductase (Bai et al., 2011) and DHAR (Bai et al., 2011) which are positively regulated by S-nitrosylation.

The novel targets were searched to detect other redox based PTMs using RedoxDB (http://biocomputer.bio.cuhk.edu.hk/ RedoxDB/index.php), a database of protein oxidative modifications. No other redox modification was identified supporting that these targets are not yet reported and are novel in plants.

To conclude, an increase in the NO production in cold suggested its role in maintaining cellular redox homeostasis in *B. juncea*. Cold induced NO reacts with low molecular weight thiols and promotes SNOs formation leading to S-nitrosylation. The fact that 17 new S-nitrosylated targets (4 GSNO treated and 13 cold responsive) were identified, which were not detected in crude (Abat and Deswal, 2009) suggest that these targets were more accessible for the purification and MS identification after RuBisCO depletion. The identified targets belong to multiple plant responses including redox homeostasis, glucosinolate hydrolysis pathway, stress signaling and Calvin cycle as described in **Figure 8**. Thus, indicated the role of accumulated NO in orchestrating these cellular responses through S-nitrosylation. Therefore, RuBisCO depletion is suitable for downstream proteomic analysis and could be used for the detection of other PTMs of cold responsive proteins that possibly are difficult to detect due to the abundance and fragmentation of RuBisCO in cold.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2013.00342/abstract

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The role of nitric oxide in the interaction of *Arabidopsis thaliana* with the biotrophic fungi, *Golovinomyces orontii* and *Erysiphe pisi*

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Powdery mildews are a diverse group of pathogenic fungi that can infect a large number of plant species, including many economically important crops. However, basic and applied research on these devastating diseases has been hampered by the obligate biotrophic lifestyle of the pathogens, which require living host cells for growth and reproduction, and lacking genetic and molecular tools for important host plants. The establishment of *Arabidopsis thaliana* as a host of different powdery mildew species allowed pursuing new strategies to study the molecular mechanisms governing these complex plant–pathogen interactions. Nitric oxide (NO) has emerged as an important signaling molecule in plants, which is produced upon infection and involved in activation of plant immune responses. However, the source and pathway of NO production and its precise function in the regulatory network of reactions leading to resistance is still unknown. We studied the response of *Arabidopsis thaliana* to infection with the adapted powdery mildew, *Golovinomyces orontii* (compatible interaction) and the non-adapted, *Erysiphe pisi* (incompatible interaction). We observed that NO accumulated rapidly and transiently at infection sites and we established a correlation between the resistance phenotype and the amount and timing of NO production. *Arabidopsis* mutants with defective immune response accumulated lower NO levels compared to wild type. Conversely, increased NO levels, generated by treatment with chemicals or expression of a NO-synthesizing enzyme, resulted in enhanced resistance, but only sustained NO production prevented excessive leaf colonization by the fungus, which was not achieved by a short NO burst although this reduced the initial penetration success. By contrast, lowered NO levels did not impair the ultimate resistance phenotype. Although our results suggest a function of NO in mediating plant immune responses, a direct impact on pathogen growth and development cannot be excluded.

Keywords: disease resistance, plant defense signaling, plant immunity, plant-microbe interaction, powdery mildew, *Golovinomyces orontii, Erysiphe pisi*

INTRODUCTION

The sessile lifestyle of plants makes it impossible for them to escape from environmental pressures. To avoid biotic stresses and colonization by microbial pathogens, such as fungi, bacteria, or viruses, plants have evolved a multitude of rapid and efficient defense mechanisms. They are guided by the ability to sense pathogen attacks and to translate this perception into an adaptive defense response. Following the detection of a pathogen *via* highly conserved microbe- or pathogen-associated molecular pattern (MAMPs or PAMPs), such as elicitor-active epitopes of bacterial flagellin (flg22) or fungal chitin, and the corresponding plasma membrane-localized pathogen pattern recognition receptors (PRR), numerous signaling molecules are released, including reactive oxygen species (ROS), calcium ions, salicylic acid (SA), jasmonic acid (JA), and nitric oxide (NO), which are thought to mediate the activation of powerful immune responses (Chisholm et al., 2006; Jones and Dangl, 2006; Boller and Felix, 2009). This PAMP-triggered immunity directed against non-adapted pathogens is also referred to basal or non-host resistance. As a mechanism to counteract plant defense mechanisms, host-adapted pathogens have acquired the capacity to escape from recognition and/or to produce effectors that suppress PRR-triggered plant defenses (Göhre and Robatzek, 2008; Deslandes and Rivas, 2012; Rafiqi et al., 2012). Plants in turn evolved a second system of immune sensors, so-called resistance (R) proteins that are localized inside plant cells and recognize pathogen effectors thereby activating an even stronger immune response (Takken and Goverse, 2012). This effector-triggered immunity shares numerous signaling and downstream components with PAMP-triggered immunity (Chisholm et al., 2006; Jones and Dangl, 2006). R protein-mediated, effector-triggered immunity typically involves defense gene activation and the hypersensitive cell death response (HR) at the site of attempted host colonization (Stuible and Kombrink, 2004; Williams and Dickman, 2008; Coll et al., 2011). The outlined dual plant defense system provides resistance against a wide variety of pathogens and only a few adapted pathogens can

successfully circumvent or suppress both defense layers and cause disease.

The causal agent of the powdery mildew disease encompasses a diverse range of pathogenic fungi (order Erysiphales) that are widespread, obligate biotrophic plant pathogens colonizing a large number of different plant species, including many economically important crops (Micali et al., 2008). With the relatively recent identification of powdery mildew species that are pathogenic on *Arabidopsis thaliana*, additional tools and experimental strategies have become available to study these complex pathogens and their interaction with this model host plant. This includes structural and functional changes that occur during host colonization, mechanisms of defense signaling/initiation and identification of genetic components responsible for compatibility and incompatibility, which may help to develop successful crop protection strategies and new agricultural practices (Micali et al., 2008).

For successful host colonization, powdery mildew conidiospores germinating on the leaf surface have to breach the epidermal cell walls, which is the first critical step of the infection process and requires formation of the appressorium and infection peg. Subsequently, the plant plasma membrane invaginates and the haustorium develops, which finally forms as branched unicellular body and functions as the intracellular feeding structure (Koh et al., 2005; Micali et al., 2008). Such established fungus can form colonies and complete the life cycle by producing conidiophores and spores for new infection. Few powdery mildew species are able to infect *Arabidopsis thaliana*, including *Golovinomyces cichoracearum* and *Golovinomyces orontii*, which are pathogens of cucurbits and crucifers (Plotnikova et al., 1998; Saenz and Taylor, 1999; Vogel and Somerville, 2000). By contrast, *Arabidopsis* is resistant to non-adapted powdery mildews, such as *Blumeria graminis* f. sp. *hordei* (pathogenic on barley) or *Erysiphe pisi* (pathogenic on pea), and this non-host resistance is readily detectable at the penetration stage by arrest of most host cell entry attempts (usually >80%). Rare cases of haustorium formation are usually accompanied by timely callose encasement and the HR of attacked epidermal cells, which prevents further fungal development (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006; Hardham et al., 2007). Thus, non-adapted powdery mildews fail to complete their life cycle on *Arabidopsis*.

Genetic analyses identified components required for non-host resistance against powdery mildew. For example, forward genetic screens yielded four *Arabidopsis* mutants, (*pen1* through *pen4*) showing enhanced penetration rates, indicating that the corresponding wild type genes are essential for the non-host resistance phenotype (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). *PEN1* encodes a syntaxin (SYP121) that mediates fusion of secretory vesicles with the plasma membrane, whereas the products of *PEN2*, a glycosyl hydrolase, and *PEN3*, an ATP-binding cassette (ABC) transporter, are predicted to load secretory vesicles with toxic compounds (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006; Micali et al., 2008). Thus, the cooperative action of PEN proteins contributes to pre-invasion/penetration resistance. In addition, post-invasion defense mechanisms restrict pathogen growth after haustorium formation. Genes encoding ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4), and SENESCENCE ASSOCIATED GENE 101 (SAG101) are essential defense components required for basal defense and execution of race-specific resistance mediated by a subset of *R* genes (Wiermer et al., 2005; Dodds and Rathjen, 2010). In the *eds1*, *pad4*, and *sag101* mutants the penetration rates of powdery mildews were not significantly different from the wild type, whereas in the double mutants *pen2 eds1* and *pen2 pad4* the non-adapted fungus was able to develop secondary hyphae while the HR occurred less frequently; in the triple mutant *pen2 pad4 sag101* non-host resistance was effectively abolished and the fungus could form microcolonies and complete its life cycle (Lipka et al., 2005; Stein et al., 2006). Thus, the removal of both defense layers, the PEN-mediated penetration resistance and the EDS1/PAD4-controlled post-invasion resistance makes *Arabidopsis* fully susceptible to non-adapted powdery mildews such as *E. pisi* (Lipka et al., 2005; Stein et al., 2006).

Biochemical and molecular analyses, complementing the genetic approaches, demonstrated that SA, JA, and ethylene signaling components could contribute to powdery mildew resistance (Reuber et al., 1998; Ellis et al., 2002; Zimmerli et al., 2004; Liu et al., 2005). In addition, the free radical NO has emerged as a signaling molecule in plant defense and its rapid production is strongly triggered after infection of plants with diverse pathogens (Delledonne et al., 1998; Leitner et al., 2009; Bellin et al., 2012). In fact, NO mediates signaling during numerous physiological processes and stress responses (Besson-Bard et al., 2008), but notably it participates, cooperatively with ROS, in the activation of HR cell death during incompatible plant-pathogen interactions (Delledonne et al., 1998; Zeier et al., 2004; Yoshioka et al., 2011). The formation of NO during plant defense frequently shows a biphasic temporal pattern, with a strong initial burst for a few minutes after infection or elicitor treatment, which is followed by a second sustained increase for several hours, and this latter increase seems to correlates with the disease resistance phenotype (Zeier et al., 2004; Mur et al., 2006). In tomato, infection with the powdery mildew fungus, *Oidium neolycopersici,* caused a rapid NO burst in both susceptible and resistant cultivars, but a sustained NO production was only observed in resistant tomato cultivars, which occurred simultaneously with a drastic increase in ROS, followed by HR cell death of penetrated epidermal cells and retardation of pathogen growth (Mlíčková et al., 2004; Piterková et al., 2009). Similarly, infection of barley with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* resulted in a transient NO burst in epidermal cells, which preceded HR cell death (Prats et al., 2005). However, how the NO and ROS signals are integrated and how precisely they mediate disease resistance remains unknown (Yoshioka et al., 2011).

Despite extensive research efforts, the precise function of NO in the plant immune response remains enigmatic. In particular, the route(s) of NO production in plants are still not unequivocally identified (Besson-Bard et al., 2008; Bellin et al., 2012). Mostly two enzymatic sources of NO are considered: (1) NO synthase (NOS; or NOS-like activity) catalyzing the NADPH-dependent oxidation of arginine as in animal cells, and (2) nitrate reductase (NR) catalyzing NO formation *via* nitrite (Yamasaki and Sakihama, 2000; Guo et al., 2003; Besson-Bard et al., 2008). In addition, NO may arise from other oxidative reactions (enzymatic and non-enzymatic) and it may be rapidly and easily converted

to other reactive nitrogen species, because NO and ROS production often occur simultaneously (Besson-Bard et al., 2008; Bellin et al., 2012). Although mutant *Arabidopsis* plants with impaired NO production are more susceptible to pathogens (Zeidler et al., 2004; Modolo et al., 2005), it is still not clear whether NO is a signal, controlling downstream defense responses, or a disease symptom functioning as a proxy of active defense, or because of its reactive nature directly impairs pathogen growth and development. By taking advantage of the genetic resources available for the model plant *Arabidopsis thaliana*, we investigated the role of NO in the interaction with the adapted and non-adapted powdery mildew fungi, *G. orontii* and *E. pisi*, respectively. Our results show that NO has the capacity to function as signal molecule and to mediate other defense responses, but an additional direct impact on pathogen growth and development cannot be excluded.

MATERIALS AND METHODS

PLANT LINES AND GROWTH CONDITIONS

In this study we used the *Arabidopsis thaliana* Col-0 genotype, the single mutants *eds1*-2 (Bartsch et al., 2006), *pen2* (Lipka et al., 2005), *nos1/noa1* subsequently referred to as *noa1* (Guo et al., 2003; Moreau et al., 2008), the double mutants *pen2 eds1*-2 (Lipka et al., 2005), *nia1 nia2* (Wilkinson and Crawford, 1993), and the *Arabidopsis* line *35S::nNOS* expressing rat neuronal NOS (nNOS) under the control of CaMV 35S promoter (Shi et al., 2012), all in the Col-0 genetic background. *Arabidopsis* seeds were surface-sterilized and placed on half-strength MS basal salt medium (Murashige and Skoog, 1962) containing 0.5% sucrose and 0.8% phytagel. After stratification for 2 days at 4°C in the dark, plates were vertically mounted under continuous yellow light for 3–4 days. Seedlings were transferred to pots with soil substrate and plants grown for 18 days at a day/night cycle of 10/14 h in a growth chamber at 22°C/20°C day/night temperature and a relative humidity of 60%.

PLANT INOCULATION AND MICROSCOPIC ANALYSIS

Four week old plants were inoculated by brushing onto rosette leaves conidia of the *Golovinomyces orontii* isolate MPIPZ or conidia of *Erysiphe pisi* isolate MPIPZ, which where propagated as previously described (Lipka et al., 2005; Göllner et al., 2008; Weßling and Panstruga, 2012). Inoculated plants were returned to the growth chamber for the indicated times. To visualize fungal structures, leaves were harvested, treated with ethanol:acetic acid 3:1 (v/v) to remove chlorophyll and stained with Coomassie Brilliant Blue as described previously (Göllner et al., 2008). Bright field images were taken with an AxioImager.A2 microscope equipped with an AxioCam HRc camera system (Carl Zeiss, Jena, Germany). All experiments were repeated twice and 5–10 images were analyzed per replicate and genotype using at least four different leaves each. A minimum of 100 fungal interaction sites was analyzed per leaf and the percentage of successful penetration events was calculated.

SPORE COUNTS

The success of leaf colonization by powdery mildews was evaluated by counting spores on inoculated leaves as previously described (Weßling and Panstruga, 2012). At 7 day post-inoculation, four leaves were harvested per genotype, submerged in 5 ml water and spores were released by thoroughly vortexing. The solution was filtered through Miracloth (Merck, Darmstadt, Germany) to remove large debris and spores were counted in a Neubauer hemocytometer (Marienfeld, Lauda-Königshofen, Germany). Spore counts were normalized to the leaf fresh weight.

DETERMINATION OF NO CONTENT

The intracellular NO level was determined by using the cell-permeable, fluorescent probe diaminofluorescein-FM diacetate (DAF-FM DA; Sigma-Aldrich, Taufkirchen, Germany), which after conversion by cytosolic esterases to DAF-FM can rapidly react with NO to form the corresponding green fluorescent triazole (DAF-FM T; Suzuki et al., 2002; Gould et al., 2003). Leaves were infiltrated with 10 mM Tris (pH 6.5) containing 10 μM DAF-FM DA (added from a 10 mM stock in DMSO) for 30 min in the dark, rinsed with water and mounted on microscopic slides. Specimen were examined with a confocal laser scanning microscope LSM 510 Meta (Zeiss, Oberkochen, Germany) equipped with an argon mixed gas laser and a filter set (excitation 488 nm, emission 515 nm) for detection of green DAF-FM T fluorescence. Serial confocal optical sections were taken at a step size of 1 μm and these Z-stacks, reconstructed into three-dimensional images, were used to quantify the NO-specific fluorescence at infection sites within areas defined by circles of approximately 50 μm in diameter by determining pixel densities with the open source software Image-J[1]. Parameters for confocal microscopy, in particular laser and detector settings, were identical for all experiments and appropriate control samples were always included. To verify that the recorded increase in fluorescence is dependent on NO accumulation, we pretreated leaves with NO scavenger (e.g., 200 μM cPTIO, see below) prior infection, which in all cases abolished DAF-FM-based fluorescence. Auto fluorescence at infection sites of control leaves was also recorded and subtracted from all experimental samples. For each data point a minimum of 20 infection sites from four different leaves taken from two different plants was analyzed and each experiment was repeated twice.

TREATMENT WITH CHEMICALS

To conditionally modulate endogenous NO levels, leaves were treated with various chemicals known to release NO, such as 200 μM *S*-nitrosoglutathione (GSNO) or 100 μM *S*-nitroso-N-acetyl-D-penicillamine (SNAP), or compounds scavenging NO or impairing its formation, such as 200 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), 100 μM L-N^{ω}-nitro-arginine methyl ester (L-NAME) or 100 μM okadaic acid (OA). All compounds (obtained from Sigma-Aldrich, Taufkirchen, Germany) were dissolved in DMSO (10 μM) and the indicated, effective working solutions in 10 mM $MgCl_2$ freshly prepared immediately before infiltration into leaves with a syringe. Plants were incubated with chemicals for 2 h before inoculation with the powdery mildews.

[1] http://rsb.info.nih.gov/ij/

QUANTIFICATION OF SALICYLIC ACID

Salicylic acid (SA) content in leaves was quantified as previously described (Straus et al., 2010). SA was extracted from 100 to 150 mg plant material in 1 ml chloroform/methanol/water (1:2:0.3) containing 160 pmol 2-hydroxybenzoic-3,4,5,6-d_4 acid (SA-d_4; Campro Scientific, Berlin, Germany) as internal standard. After shaking for 10 min at 70°C samples were centrifuged and re-extracted with 0.5 ml chloroform/methanol (1:2). After phase separation through the addition of 0.5 ml H_2O the polar extract was dried. Samples were dissolved in 1 ml sodium acetate (pH 5) and divided equally for total and free SA analysis. For total SA, samples were treated with almond β-glucosidase (Sigma-Aldrich, Taufkirchen, Germany) for 3 h at 37°C. Both, total and free SA samples were acidified with 30 µl 10% trifluoroacetic acid (TFA) and extracted twice with 0.6 ml ethyl acetate/hexane (3:1). Following evaporation of organic solvents, analytes were derivatized with 80 µl pyridine/N-methyl-N-(trimethylsilyl)trifluoroacetamide (1:1; Sigma-Aldrich, Taufkirchen, Germany) and 1 µl was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent Technologies)[2]. Masses of SA-d_4 (*m/z* 271) and SA (*m/z* 267) were detected by selected ion monitoring and quantified using the Chemstation software from Agilent.

QUANTITATIVE REAL-TIME PCR

Relative transcript levels of PR1 were determined by quantitative real-time PCR (qRT-PCR) according to established protocols (Schmittgen and Livak, 2008; Weßling and Panstruga, 2012). Total RNA was extracted from 100 mg leaf issue and reverse transcribed to generate first-strand cDNA with the Super-Script First-Strand Synthesis System for RT-PCR (Invitrogen, Darmstadt, Germany) using oligo(dT) and random hexamer primers according to the manufacturer's protocol. All qPCR assays were performed with cDNA corresponding to 100 ng RNA using the iQ™ SYBR® Green Supermix Kit (Biorad)[3] on the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, München, Germany). We used gene-specific primers at a final concentration of 0.1 µM and expression of the actin gene (At3g18780) served as control (PR1-forward: TTCTTCCCTCGAAAGCTCAA, PR1-reverse: AAGGCCCACCAGAGTGTGTATG; actin-forward: CGGTAACATTGTGCTCAGTGGTGG; actin-reverse: CAACGACCTTAATCTTCATGCTGC). qPCR assays were carried out in three technical replicates per sample according to the following conditions: denaturation at 95°C for 2 min, 40 repeats at 95°C for 20 s, 56°C for 30 s, and 72°C for 25 s. Relative expression levels were calculated using the $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008) and normalized to the expression in uninfected control plants (0 hpi).

RESULTS

NO ACCUMULATION IN ARABIDOPSIS LEAVES UPON INOCULATION WITH POWDERY MILDEWS

To monitor NO production during the interaction of *Arabidopsis thaliana* with powdery mildew fungi, we used the cell-permeable dye DAF-FM DA (4-amino-5-methylamino-2,7-difluorofluorescein-FM diacetate), which is an established, specific probe for the detection of intracellular NO (Suzuki et al., 2002; Gould et al., 2003), in combination with confocal laser-scanning microscopy. When loaded into plant cells, DAF-FM DA is converted by cytosolic esterase to DAF-FM, which can react with N_2O_3, originating from oxidation of NO, to form the green fluorescent DAF-FM triazole derivative. When *Arabidopsis* plants (Col-0) were inoculated with the adapted powdery mildew *G. orontii*, rapid and localized NO accumulation was demonstrated by confocal laser scanning microscopy, which is restricted to few directly affected cells (**Figure 1A**). Quantitative analysis revealed a strong increase in NO amounts at the infection sites reaching maximum levels at 8 h post-inoculation followed by a rapid decrease thereafter (**Figure 1B**). The peak of NO formation coincided in timing with appressoria formation by *G. orontii* primary hyphae on the leaf surface, which initiates breaching of epidermal cell walls and precedes the formation of infection hyphae. Plants inoculated with the non-adapted powdery mildew fungus, *E. pisi*, showed a similar spatial pattern of NO formation, again

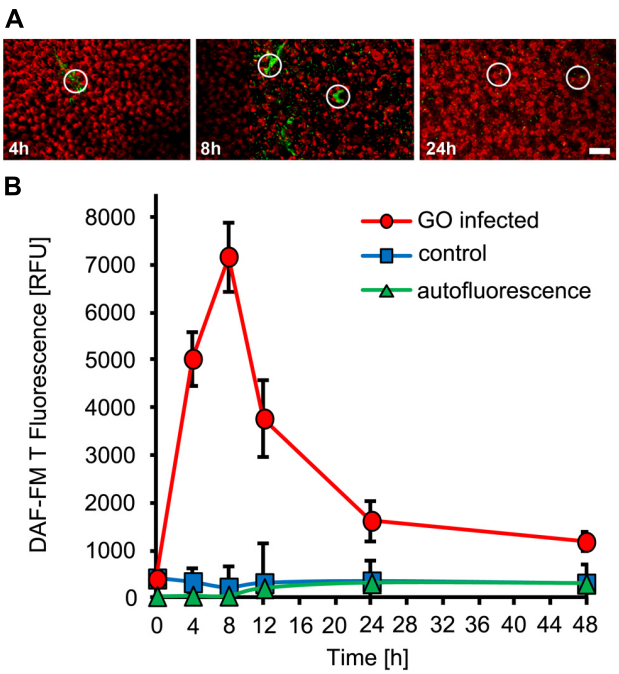

FIGURE 1 | NO accumulation in *Arabidopsis* leaves upon inoculation with the adapted powdery mildew fungus, *Golovinomyces orontii*. Leaves of *Arabidopsis thaliana* Col-0 were harvested at the indicated times after inoculation and used to detect intracellular NO by infiltration of the NO sensitive dye DAF-FM DA. **(A)** Time series of confocal images (taken at 4, 8, and 24 h post-inoculation) showing focused NO accumulation, as indicated by the green fluorescence, at the powdery mildew infection sites (white circles). The red color is due to chlorophyll fluorescence. **(B)** Time course of NO accumulation at fungal infection sites (red circles) and corresponding areas of non-infected control leaves (blue squares). NO was quantified by integrating the pixel intensity of green DAF-FM T fluorescence in three-dimensional optical reconstructions of infections sites (area defined by circles). Autofluorescence at infection sites was quantified without prior staining with DAF-FM DA (green triangles). Each data point reflects the mean (±SD) of 20 infection sites taken from four different leaves of two different plants. Bar = 50 µM.

[2]http://www.agilent.com/

[3]http://www.bio-rad.com/

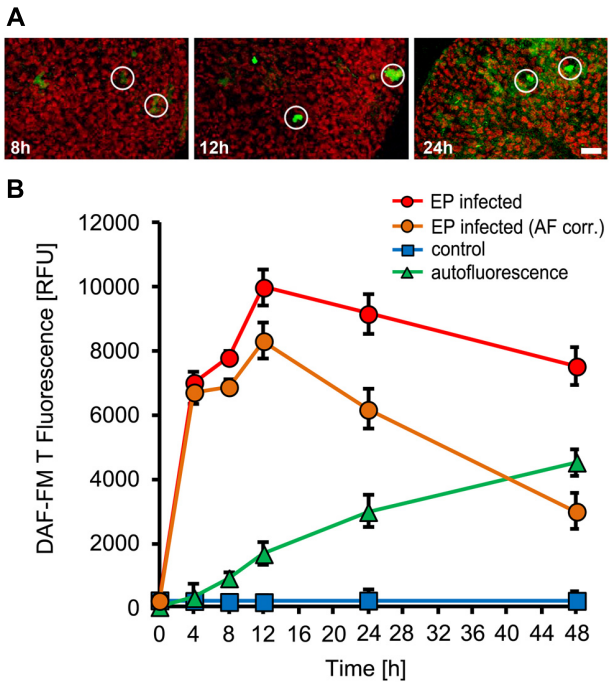

FIGURE 2 | NO accumulation in *Arabidopsis* leaves upon inoculation with the non-adapted powdery mildew fungus, *Erysiphe pisi*. Leaves of *Arabidopsis thaliana* Col-0 were harvested at the indicated times after inoculation and used to detect intracellular NO by infiltration of the NO sensitive dye DAF-FM DA. **(A)** Confocal images of powdery mildew infection sites (white circles) taken at 8, 12, and 24 h post-inoculation. **(B)** Time course of NO accumulation at infection sites (red circles) and in non-infected control leaves (blue squares). Autofluorescence at infection sites was quantified without prior DAF-FM DA staining (green triangles) and these values were used to correct NO levels (orange circles). All data represent the mean (±SD) of 20 infection sites taken from four different leaves of two different plants. Bar = 50 μM.

restricted to few cells around infection sites (**Figure 2A**). However, the time course was delayed (maximum at 12 hpi) and the overall amounts of NO accumulating at infection were slightly higher when compared to *G. orontii* infection (**Figure 2B**). The incompatible interaction of *Arabidopsis* with non-adapted *E. pisi* is characterized by the development of rapid HR cell death of infected cells, which is associated with strong autofluorescence and therefore may interfere with NO detection and systematically distort its quantification. We examined the autofluorescence in infected tissue without DAF-FM staining and observed a continuous increase over time, which was used to correct the determined NO levels accordingly (**Figure 2B**). Obviously, NO quantification is primarily distorted at late time points (**Figure 2B**). By contrast, only low values of autofluorescence were recorded following inoculation with *G. orontii* and hence, the NO quantification was not affected (**Figure 1B**). From these infection studies it is evident that NO accumulation is a rapid, localized defense response and the rapid decline of initially high values in the compatible interaction of *Arabidopsis* with *G. orontii* may suggest that the adapted powdery mildew has developed strategies to remove NO or suppress its excessive accumulation.

NO FORMATION IN ARABIDOPSIS MUTANTS WITH IMPAIRED DISEASE RESISTANCE

To further explore the potential function of NO in plant immunity, we determined NO formation in *Arabidopsis* mutants that are impaired in their defense. First, we tested the *Arabidopsis pen2* mutant, which is compromised in penetration resistance toward non-adapted powdery mildews, such as *E. pisi*. In *pen2* NO formation essentially followed a similar time course as in wild type plants, with the exception that up to 12 h the absolute amounts are 25–30% lower (**Figure 3**). Since at 24 h post-inoculation the penetration frequency of *E. pisi* on *pen2* plants is drastically increased (60–80% of the interaction sites), this early reduction in NO correlates with and may be responsible for the complete loss of resistance and successful invasion of the mutant (**Figure 5A**; Lipka et al., 2005). The complete susceptibility of *Arabidopsis* toward adapted powdery mildew *G. orontii* is not further enhanced in the *pen2* mutant (not shown).

Second, in the *eds1* mutant penetration resistance toward *E. pisi* is not impaired, but epiphytic hyphal growth, which occurs later during this interaction, is substantially increased, when scored at 7 days post-inoculation (Lipka et al., 2005). NO formation in *eds1* plants was unaffected during the early stages of interaction with *E. pisi* in comparison to wild type plants (**Figure 3**). However, drastically reduced NO levels were observed at 24–48 h post-inoculation, amounting to 40–50 % of wild type levels. Thus, suppression of NO formation or its removal at late infection stages may be causal for subsequent successful colonization of mutant tissue by the non-adapted powdery mildew fungus. The NO accumulation pattern in the *eds1 pen2* double mutant exactly matches the combined patterns of both single mutants, with reduced NO levels throughout the time period analyzed (**Figure 3**). Again, this correlates with

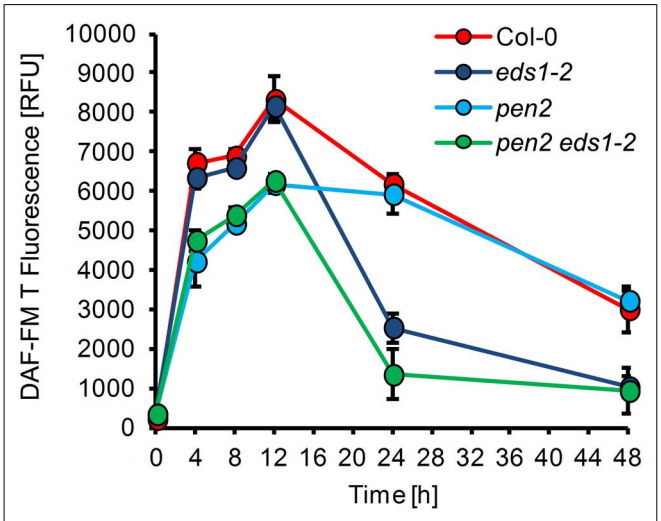

FIGURE 3 | NO formation in *Arabidopsis* mutants with impaired disease resistance. Leaves of different *Arabidopsis thaliana* genotypes, Col-0 (red), *eds1-2* (purple), *pen2* (blue), *pen2 eds1-2* (green), were inoculated with the non-adapted powdery mildew *Erysiphe pisi* and harvested at the indicated times for quantification of NO formation by integration of DAF-FM T fluorescence at infection sites. (For experimental details, see **Figure 1**). Values represent the mean (±SD) of 20 infection sites taken from four different leaves of two different plants.

impaired penetration resistance and even further enhanced epiphytic fungal growth on the leaf surface, resulting in microcolony formation as reported previously (Lipka et al., 2005).

POWDERY MILDEW INFECTION OF ARABIDOPSIS MUTANTS WITH IMPAIRED NO PRODUCTION

In order to identify the metabolic route(s) of powdery mildew-induced NO formation, we used two *Arabidopsis* mutants with impaired capacity to synthesize NO. First, the contribution of NR was evaluated by analysis of the *nia1 nia2* double mutant, which is defective in both genes encoding active NR, NIA1, and NIA2 (Wilkinson and Crawford, 1993; Desikan et al., 2002). This mutant showed strongly reduced NO production upon infection with necrotrophic fungal pathogens and bacteria, such as *Botrytis cinerea, Sclerotinia sclerotiorum,* and *Pseudomonas syringae* (Modolo et al., 2006; Asai et al., 2010; Oliveira et al., 2010; Perchepied et al., 2010), but when inoculated with *E. pisi*, the pattern of NO formation in was indistinguishable from wild type plants (**Figure 4**). This result indicates that in the *Arabidopsis nia1 nia2* double mutant NO synthesis upon powdery mildew infection proceeds *via* an NR-independent pathway. Likewise, the resistance phenotype of the *nia1 nia2* double mutant was also not different from wild type plants, both allowing a rate of 26% successful penetration events (**Figure 5A**). This similarity in phenotypic appearance is also obvious from inspection of the tissue under the microscope (**Figure 5B**) and it is in accordance with the unaltered NO levels.

Second, we determined whether NO synthesis may originate from L-arginine *via* a NOS-like activity, which previously was believed to exist in plants and contribute to pathogen-induced NO formation (Guo and Crawford, 2005; Corpas et al., 2009; Asai et al., 2010). Although the *noa1* mutant is defective in a plastidic GTPase rather than NOS (Moreau et al., 2008; Gas et al., 2009), it shows reduced NO levels after bacterial infection or elicitor treatment (Delledonne et al., 1998; Zeidler et al., 2004). We found that upon inoculation with *E. pisi* the *noa1* mutant accumulated approximately 20–30% less NO in comparison to wild type plants (**Figure 4**). At the same time, the penetration rate of the powdery mildew fungus on the mutant increased slightly, but not significantly (from 26 to 36% compared to wild type), and also histological differences were not apparent (**Figures 5A** and **B**). The nearly unimpaired NO formation in the *nia1 nia2* double and *noa1* single mutant indicates that NO synthesis in *Arabidopsis* proceeds *via* a yet unknown route.

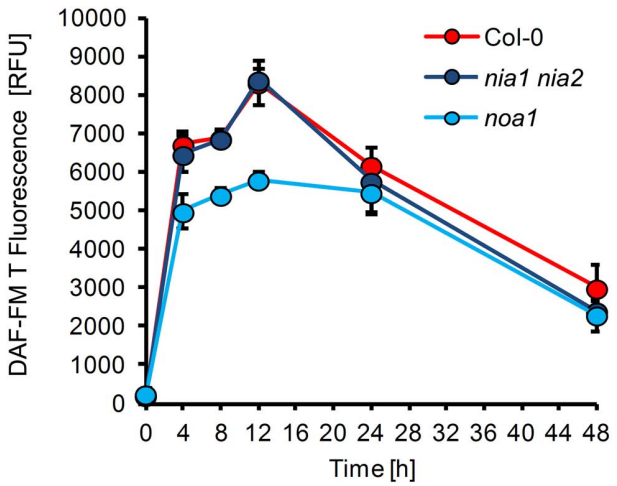

FIGURE 4 | NO formation in *Arabidopsis* mutants defective in putative NO synthesis pathways. Leaves of different *Arabidopsis thaliana* genotypes, Col-0 (red), *nia1 nia2* (purple), *noa1* (blue), were inoculated with the non-adapted powdery mildew *Erysiphe pisi* and harvested at the indicated times for quantification of NO formation by integration of DAF-FM T fluorescence at infection sites. (For experimental details see legend to **Figure 1**). Values represent the mean (±SD) of 20 infection sites taken from four different leaves of two different plants.

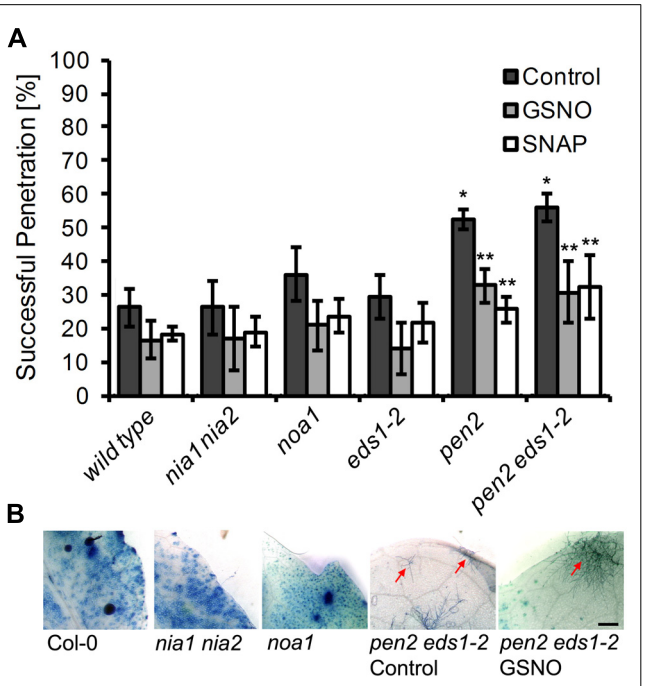

FIGURE 5 | Disease resistance phenotype of different *Arabidopsis* mutants to infection with *Erysiphe pisi*. **(A)** Quantitative analysis of host cell entry (penetration rates), determined 48 h post-inoculation with *Erysiphe pisi* (black bars). The same analysis was carried out with leaves that were infiltrated with NO donors, 200 μM GSNO (gray bars) or 100 μM SNAP (white bars) 2 h prior to inoculation with *E. pisi* spores. Data represent the mean (±SD) of at least six leaves taken from two different plants. One asterisk indicates a significant difference ($p < 0.01$) between mutant and wild type plants, two asterisks indicates a significant difference ($p < 0.01$) between control and NO donor treatment. **(B)** Representative micrographs of infected leaves, harvested at 7 days post-inoculation, following staining with Coomassie Brilliant Blue to visualize fungal structures and host cells that have undergone HR cell death. The *Arabidopsis* mutant *noa1* and the double mutant *nia1 nia2* show no phenotypic difference to wild type plants (Col-0), whereas the double mutant *pen2 eds1-2* shows a lower frequency of HR cell death and sporadic microcolony formation (red arrow), which is not affected by pre-treatment with GSNO/SNAP. Bar = 1 mm.

IMPACT OF CHEMICALLY ALTERED OF NO LEVELS ON POWDERY MILDEW INFECTIONS

Since the available mutants did not show significant alterations in cellular NO levels, we used a chemical approach to study the impact

of NO accumulation on powdery mildew infections. Therefore, we first treated plants for 2 h with the NO scavenger cPTIO, the NOS inhibitor L-NAME, or the NR inhibitor OA, followed by inoculation with the non-adapted powdery mildew *E. pisi*. However, none of the tested compounds caused a significant increase in penetration frequency (**Figure 6**), suggesting that NO does not contribute to disease resistance and plant colonization by *E. pisi* is rather limited by other defense components. Conversely, plants treated with the NO donors GSNO or SNAP for 2 h prior to inoculation by *E. pisi* showed clearly reduced penetration rates, which is true for all plant genotypes tested, including wild type, *nia1 nia2*, and *noa1* (**Figure 5A**). In fact, both NO donors reduced the initial penetration phenotypes of the *pen2* and *pen2 eds1* mutant back to wild type levels, which could be explained by NO directly impairing fungal viability or indirectly enhancing other defense responses. However, the low frequencies of HR cell death and microcolony formation by *E. pisi* on the *pen2 eds1* double mutant was not affected by treatment with GSNO (**Figure 5B**).

We also tested the impact of NO donors on infection of *Arabidopsis* by the adapted powdery mildew *G. orontii*. Treatment of leaves with GSNO or SNAP caused a significant reduction in penetration frequency (from 88 to 67 and 53%, respectively) as determined 2 days after inoculation (**Figure 7A**). However, this treatment did not affect the final outcome of this compatible interaction; when spores of the colonized leaves were counted 7 days after inoculation, we observed no significant differences in comparison to the control (**Figure 7B**). Both treated and untreated leaves were fully covered with sporulating *G. orontii* colonies (**Figure 7C**). One possible explanation for this result could be that NO donor treatment enhanced NO levels only for a short time period. Indeed, NO quantification revealed that GSNO-treated

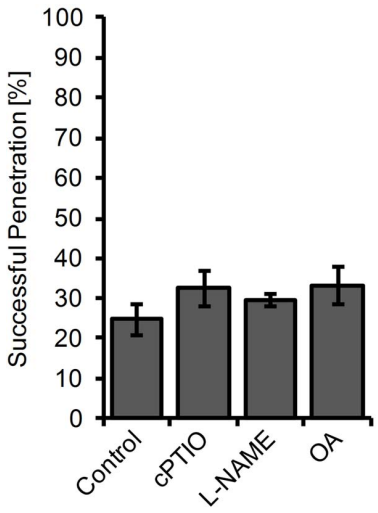

FIGURE 6 | Penetration rate of *Erysiphe pisi* on *Arabidopsis* is not affected by inhibitors of NO formation. *Arabidopsis* wild type plants were treated for 2 h with 200 μM cPTIO, 100 μM L-NAME, 100 μM OA, or DMSO (control) in 10 mM $MgCl_2$ before inoculation with the powdery mildew fungus *E. pisi* and host cell penetration was quantified 48 h post-inoculation. Data represent the mean (±SD) of all (at least 100) interactions sites analyzed on six leaves taken from two different plants.

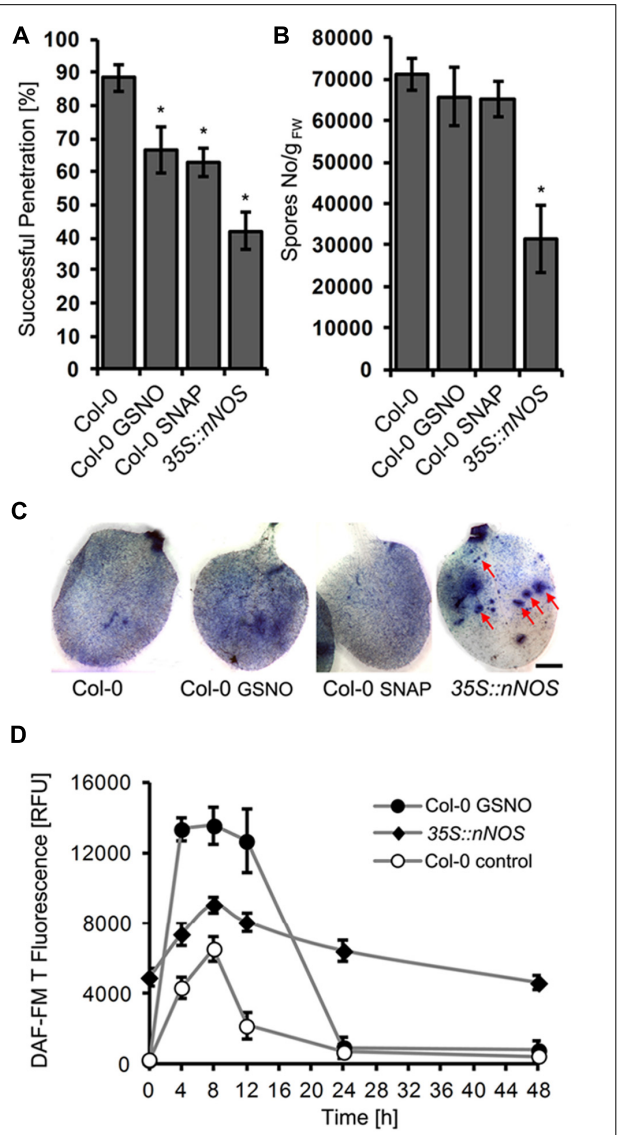

FIGURE 7 | Increased NO levels impair colonization of *Arabidopsis* by *Golovinomyces orontii*. Six week-old *Arabidopsis* wild type (Col-0) was infiltrated with NO donors, 200 μM GSNO, 100 μM SNAP, or DMSO (control) 2 h before inoculation with *G. orontii* spores, whereas the *Arabidopsis* mutant *35S::nNOS* was inoculated without prior treatment. **(A)** Quantification of host cell entry rates determined 48 h post-inoculation (hpi). Data represent the mean (±SD) of at least six leaves taken from two different plants. **(B)** Production of spores at 7 days post-inoculation (dpi) normalized to leaf fresh weight. Data represent the mean (±SD) of at least four different leaves. Asterisks in both graphs (**A** and **B**) indicate a significant difference ($p < 0.01$) of treated plants/mutant relative to untreated wild type. **(C)** Representative micrographs of infected leaves, harvested at 7 days post-inoculation (dpi), following staining with Coomassie Brilliant Blue to visualize fungal structures and host cells that have undergone HR cell death. The *Arabidopsis* wild type is covered with sporulating colonies and this phenotype is not altered by prior treatment with GSNO or SNAP. By contrast, the *35S::nNOS line* is only partially colonized and developed intensely stained lesions (HR cell death) at high frequency (red arrows). Bar = 25 mm. **(D)** Time course of NO formation in *Arabidopsis* wild type (Col-0) and *35S::nNOS* plants upon inoculation with the adapted powdery mildew fungus, *G. orontii*. NO quantification at infection sites was carried out as described in **Figure 1**. Values represent the mean (±SD) of at least 20 infection sites taken from four different leaves.

plants contained about two-fold higher NO levels at 8 h after infection with *G. orontii* in comparison to untreated control plants (**Figure 7D**). However, this increase rapidly vanished and at 24 h after inoculation, NO amounts declined to background level in both cases (**Figure 7D**).

Next we tested whether disease resistance is affected by sustained NO production. Therefore, we inoculated transgenic *Arabidopsis* plants expressing rat nNOS under the control of the CaMV 35S promoter (Shi et al., 2012). NO quantification confirmed that these plants contained drastically enhanced NO levels (compared to wild type), which transiently increased further after inoculation with *G. orontii* (**Figure 7D**). The phenotypic analysis revealed that these *35S::nNOS* plants showed a strongly reduced penetration rate after inoculation with *G. orontii* (42 vs 88% in wild type; **Figure 7A**), and also spore formation was significantly reduced (**Figure 7B**). Furthermore, the leaves of *35S::nNOS* plants were only partially colonization and unlike wild type plants developed necrotic lesions (**Figure 7C**). From these results we conclude that sustained NO formation has a positive impact on disease resistance, whereas temporal variation of NO concentrations is apparently insufficient.

IMPACT OF ENHANCED NO LEVELS ON OTHER DEFENSE RESPONSES

Having shown a resistance phenotype of NO overproducing plants, we wanted to analyze whether this NO function is directly affecting the pathogen or whether it is mediated *via* other defense responses. We therefore analyzed two typical defense marker, expression of the *PR1* gene and accumulation of SA (Glazebrook, 2005; Vlot et al., 2009). In unchallenged *35S::nNOS* plants, PR1 gene expression was about 4-fold enhanced when compared to wild type plants (**Figure 8A**), and upon *G. orontii* infection it was about 10-fold induced in both genotypes (**Figure 8A**). Quantification of SA uncovered a similar pattern. Unchallenged *35S::nNOS* plants contained about 2-fold higher concentrations of total SA, which increased about 2.5-fold upon inoculation with *G. orontii*, as in wild type plants (**Figure 8B**). From these results we conclude that NO has the capacity to function as signal molecule to mediate other defense responses; however, a direct impact on pathogen growth and development cannot be dismissed.

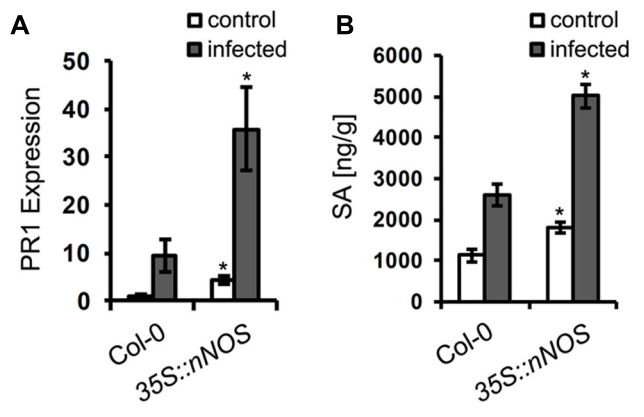

FIGURE 8 | Salicylic acid content and *PR1* gene expression are upregulated in *35S::nNOS* plants. 6-week-old *Arabidopsis* plants, wild type and *35S::nNOS*, were inoculated with *Golovinomyces orontii* and leaves harvested for analysis at 24 h post-inoculation. **(A)** Relative expression of the *PR1* gene was determined by qRT-PCR (normalized to *actin* gene expression) and the value of unchallenged Col-0 plants set to 1. **(B)** Quantification of total SA levels. All values are the mean (±SD) of six plants taken from two separate experiments. In both graphs (**A** and **B**), asterisks indicate a significant difference ($p < 0.01$) of mutant relative to wild type plants.

DISCUSSION

Initially, NO has been identified as regulator of numerous physiological responses in mammals and many years later similar biological functions of this molecule were uncovered in plants by demonstrating that it is an crucial component of the plant immune response (Delledonne et al., 1998; Durner et al., 1998). Importantly, NO participates, in cooperation with H_2O_2 (and other ROS), in activation of HR cell death in incompatible plant–pathogen interactions (Mur et al., 2006; Yoshioka et al., 2011; Bellin et al., 2012). This NO function has mainly been demonstrated when plants were infection with pathogenic bacteria, e.g., *Pseudomonas syringae* (Delledonne et al., 1998; Zeidler et al., 2004; Zeier et al., 2004; Modolo et al., 2005, 2006; Zago et al., 2006; Oliveira et al., 2010), but a contribution of rapid NO bursts to enhanced disease resistance has also been observed in various plants under attack by necrotrophic fungal pathogens, such as *Botrytis cinerea* or *Sclerotinia sclerotiorum* (Mur et al., 2006; Floryszak-Wieczorek et al., 2007; Asai and Yoshioka, 2009; Perchepied et al., 2010). By contrast, only few studies, focusing on the crop plants barley and tomato, have assessed the role of NO against biotrophic fungi such as powdery mildews (Prats et al., 2005; Piterková et al., 2009).

We wanted to elucidate the role of NO in the plant immune response toward biotrophic fungi by using the model plant *Arabidopsis thaliana* infected by the host-adapted powdery mildew *G. orontii* (compatible interaction) or the non-adapted powdery mildew *E. pisi* (incompatible interaction). We monitored NO formation with fluorescent dye DAF-FM DA, which not only allows quantification but can also provide insight into spatial accumulation patterns with cellular resolution. The specificity of DAF-FM DA for detection of NO has previously been demonstrated (Suzuki et al., 2002; Besson-Bard et al., 2008), although some caution is required to work under strictly aerobic conditions because NO reacts with the dye only in the presence of oxygen *via* the intermediate N_2O_3 (Arita et al., 2007). Applying this methodology, we could clearly show that *Arabidopsis*, similar to barley and tomato (Prats et al., 2005; Piterková et al., 2009), responds to powdery mildew infection with a rapid and transient NO accumulation, which is restricted to infection sites (**Figures 1** and **2**). While the rapid accumulation of NO was similar in both, the compatible and the incompatible interaction, differences in the duration of elevated NO levels were apparent. In leaves infected with *G. orontii*, the NO level rapidly declined after the initial burst, which could be a consequence of active defense suppression mediated by effector molecules deployed by the host-adapted powdery mildew (O'Connell and Panstruga, 2006). By contrast, NO levels remained high for an extended time period following inoculation with *E. pisi* (**Figure 2**). Interestingly, the peak of NO accumulation (at 8–12

hpi) coincided with the time reported for formation of appressoria, which is a prerequisite for breaching the plant cell and presumably this process is also tightly linked with recognition of the pathogen by the host and coordinate defense activation.

The advantage of *Arabidopsis* as experimental system is the vast availability of various biological resources, experimental tools and acquired common knowledge. Therefore, we could access different mutants with defective pathogen defense. The analysis of two *Arabidopsis* mutants, *pen2* and *eds1*, which are impaired in pre-invasion and post-invasion defense mechanisms, respectively, are also differentially affected in their capacity of NO formation. In *pen2*, the temporal pattern of NO accumulation after inoculation with *E. pisi* was not affected, but the total amount was significantly reduced, which correlates with enhanced penetration rates of the fungus (**Figures 3** and **5**), whereas extended hyphal growth and sporulation of the fungus was not supported (Lipka et al., 2005). In *eds1*, by contrast, the initial increase of NO was not affected, but a significant decrease occurred subsequently at late infection stages, which correlates with enhanced epiphytic fungal growth and formation of microcolonies (**Figures 3** and **5**), and this phenotype is further enhanced in the *pen2 eds1* double mutant (Lipka et al., 2005). Although our results may suggest that impaired resistance is the result of reduced NO accumulation, we cannot necessarily infer such causal relationship. The gene products encoded by *PEN2* (glycosyl hydrolase/myrosinase) and *EDS1* (central regulator of plant defense) are functionally well characterized and not related to nitrogen or NO metabolism (Lipka et al., 2005; Wiermer et al., 2005; Bednarek et al., 2009). In fact, the inverse relationship cannot be excluded. Reduced NO levels in *pen2* and *eds1* may be the consequence of enhanced host colonization if the pathogen, *E. pisi*, has the capacity to suppress NO formation or to decompose the molecule.

The second type of mutant we used in our studies is affected in NO biosynthesis. In fact, two enzymatic pathways for NO synthesis have been described in plants (Besson-Bard et al., 2008). The first pathway includes a cytosolic NR, which produces NO *via* nitrite, but only with low efficiency (Yamasaki et al., 1999; Yamasaki and Sakihama, 2000). The *Arabidopsis* genome contains two NR genes, *NIA1* and *NIA2*, and their participation in NO formation is supported by the abolition of NR activity and NO production in the *nia1 nia2* double mutant (Desikan et al., 2002). The mutant is also defective in nitrogen assimilation, it contains decreased levels of nitrite and amino acids, and the impaired NO formation after pathogen infection can be rescued be application of nitrite (Modolo et al., 2005, 2006). The second pathway implicates a putative NOS-like enzyme catalyzing arginine-dependent NO formation in plants, although a homolog of animal NOS has not been identified in any sequenced plant genome (Corpas et al., 2006, 2009; Besson-Bard et al., 2008; Asai and Yoshioka, 2009). However, inhibitors of animal NOS also suppress NO formation in plants (Delledonne et al., 1998) and the *Arabidopsis noa1* mutant shows reduced NO levels (Guo et al., 2003; Guo and Crawford, 2005). Previously, this mutant was considered to be impaired in NOS, but recently it was demonstrated that the defective gene encodes a functional GTPase and the reduced NO levels are an indirect consequence of the mutation, impairing chloroplast functions, and therefore NOS was renamed to NO-ASSOCIATED PROTEIN 1 (NOA1) (Moreau et al., 2008; Gas et al., 2009). In any case, the *Arabidopsis* mutant *noa1* is not only impaired in NO production but is also more susceptible to infection by diverse pathogens, including *Pseudomonas syringae, Colletotrichum orbiculare* and *Sclerotinia sclerotiorum* (Zeidler et al., 2004; Asai et al., 2008; Perchepied et al., 2010). By contrast, our results clearly show that in the *nia1 nia 2* double mutant the time course and amount of NO accumulation after inoculation with *E. pisi* is not different from the wild type and in the *noa1* mutant the amount is only slightly reduced, to about 70–80% of the wild type level (**Figure 4**). More importantly, we found no significant difference in disease resistance of both types of mutant toward *E. pisi* in comparison to wild type. These results indicate that none of the two outlined pathways (NR or NOS) seems to contribute to NO formation in *Arabidopsis* following infection by the biotrophic fungus *E. pisi*. Instead, NO may originate from a yet unknown pathway(s) or from non-enzymatic reactions (Besson-Bard et al., 2008). Furthermore, a contribution of NO to disease resistance can neither be inferred nor excluded from this mutant comparison, because the NO levels were only insufficiently altered.

The lack of additional NO-deficient mutants required alternative strategies to unveil the origin of NO and to modulate its cellular amounts. We have chosen a chemical approach to alter the plant endogenous NO-levels. Pretreatment of leaves with L-NAME, a widely used animal NOS inhibitor, which also suppresses NO synthesis in plants (Barroso et al., 1999; Rasul et al., 2012), or the NR inhibitor OA (Rockel et al., 2002), did not significantly affect plant resistance toward *E. pisi*, and likewise the NO scavenger cPTIO had also no effect (**Figure 6**). This is in accordance with the infection phenotypes of the NO-deficient mutants, *nia1 nia2* and *noa1*, collectively suggesting that NO is not involved in mediating immune responses to biotrophic pathogens. However, the opposite approach, increasing endogenous NO level by treatment of leaves with NO donors GSNO or SNAP prior to infection with *E. pisi* resulted in enhanced penetration resistance (**Figure 5**). This response was observed in all *Arabidopsis* genotypes infected with the non-adapted powdery mildew *E. pisi*, and the penetration rates of the adapted powdery mildew *G. orontii* were also significantly impaired by NO donor treatment (**Figure 7A**). However, this increased penetration resistance, manifested at 2 days post-inoculation, did not translate into post-invasion resistance and, despite pretreatment, *G. orontii* was able to complete its life cycle and colonize the host, as evident at 7days post-inoculation (**Figures 7B** and **C**). The quantification of NO in these NO donor-treated plants revealed a transiently enhanced accumulation upon infection, which could explain the reduced penetration frequency. For *Colletotrichum coccodes* it is documented that NO delays *in vitro* germination of conidia (Wang and Higgins, 2005) and for the tomato powdery mildew *Oidium neolycopersici* it has been shown that the transition from conidia to hyphae is sensitive to NO (Piterková et al., 2011). However, our results also indicate that after the initial burst, when NO had declined to background level, *G. orontii* could obviously resume growth and eventually colonized the whole leaf (**Figure 7C**). Thus, *G. orontii* can apparently cope with NO and even an active role in modulating its amount by degradation/decomposition or synthesis cannot be dismissed. Indeed, several fungi have been shown to produce NO *in vitro* and

in vivo, including *Pythium* sp., *Botrytic* sp., *Fusarium* sp., *Blumeria graminis* and *Magnaporthe oryzae*, but the functional significance is unknown and possible routes of synthesis unresolved (Conrath et al., 2004; Prats et al., 2008; Samalova et al., 2013).

Although NO can also stimulate fungal development and/or drive the infection process, our results, and most of the published data, rather support an adverse effect of NO on fungal growth. This is most evident from the analysis of the *35S::nNOS* expressing *Arabidopsis* line, which contained constitutively enhanced NO levels, in contrast to the transient burst that was achieved upon treatment with NO donors, and which rendered the plant more resistant to infection by *G. orontii* (**Figure 7**). This transgenic line was previously shown to also display enhanced resistance to *P. syringae* and various abiotic stresses (Shi et al., 2012). Since this enhance resistance is associated with accumulation of SA and enhanced expression of defense marker genes such as PR1 (**Figure 8**), and many others (Shi et al., 2012), it can speculated that fungal growth restriction is the result of multiple plant defense components that are mediated by NO. However, a direct impact of NO on fungal growth and development is not excluded.

The production of NO is a conspicuous feature of the plant immune response and many details of its synthesis are still hidden in the haze. By contrast, concerning the function of NO a picture is emerging that involves *S*-nitrosylation of cysteine thiols as pivotal regulatory mechanism for the activation of plant defense responses (Besson-Bard et al., 2008; Leitner et al., 2009; Bellin et al., 2012). Among the numerous proteins that are *S*-nitrosylated several important regulators of plant defense were identified, including the transcriptional co-regulator NON-EXPRESSOR OF PR1 (NPR1) mediating SA-dependent defense activation (Tada et al., 2008; Lindermayr et al., 2010), the SA-BINDING PROTEIN 3 (SABP3) involved in SA signaling and expression of resistance against pathogen infection (Wang et al., 2009), and the NADPH oxidase RBOHD mediating HR cell death development by synthesis of ROS (Yun et al., 2011). The activity of all these proteins was affected by *S*-nitrosylation, and although this modification mechanism involves additional components such as glutathione (GSH), GSNO, ROS, and other redox mediators, it is obvious that NO has the potential to play a crucial role in defense signaling. Therefore, it is not surprising that constitutively enhanced NO production leads to defense activation, e.g., SA accumulation and PR1 gene expression, and eventually results in elevated disease resistance, as we demonstrated for the *Arabidopsis 35S::nNOS* line (**Figure 8**) and others previous reported (Shi et al., 2012).

In conclusion, NO plays a pivotal role in the immune response of plants to attack by diverse microbial pathogens, not only bacteria and necrotrophic fungi (as previously reported), but also biotrophic powdery mildews (as our results show). We demonstrated that a key feature of pathogen-induced NO formation is the rapid and transient accumulation and by extending the time period of elevated NO levels by chemical or genetic manipulation, enhanced disease resistance could be achieved. However, the molecular mechanism of this NO bioactivity is still largely unknown and this is also true for the route(s) of NO synthesis during plant–microbe interactions. Our analysis of two NO-deficient *Arabidopsis* mutants (*nia1 nia2* and *noa1*) excluded NO production *via* the known NR and/or NOS-like pathways. Clearly, there is a need for additional genetic resources to unravel NO biosynthesis and function and therefore we initiated a genetic screen in search for new and/or alternative components that should help to uncover the origin and potential targets of this important signaling component.

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Nitric oxide-sphingolipid interplays in plant signaling: a new enigma from the Sphinx?

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Nitric oxide (NO) emerged as one of the major signaling molecules operating during plant development and plant responses to its environment. Beyond the identification of the direct molecular targets of NO, a series of studies considered its interplay with other actors of signal transduction and the integration of NO into complex signaling networks. Beside the close relationships between NO and calcium or phosphatidic acid signaling pathways that are now well-established, recent reports paved the way for interplays between NO and sphingolipids (SLs). This mini-review summarizes our current knowledge of the influence NO and SLs might exert on each other in plant physiology. Based on comparisons with examples from the animal field, it further indicates that, although SL–NO interplays are common features in signaling networks of eukaryotic cells, the underlying mechanisms and molecular targets significantly differ.

Keywords: sphingolipids, ceramides, long chain bases, nitric oxide, plant, signaling, abiotic and biotic stresses

INTRODUCTION

Nitric oxide (NO) is a pleiotropic actor of signaling cascades in eukaryotes (Baudouin, 2011; Martínez-Ruiz et al., 2011). The last 15 years have provided a plethora of examples for the involvement of NO essentially at all stages of plant development or in response to most environmental cues (Baudouin, 2011; Mur et al., 2013). *De facto* cardinal questions such as the origin, mode of action, or integration of NO signal into regulatory networks became of broad interest for plant biologists (Besson-Bard et al., 2008; Mur et al., 2013). The complex chemistry of NO enables its reactivity toward an array of biological molecules including proteins, DNA, and lipids (Calcerrada et al., 2011). In particular specific protein targets that undergo NO-based post-translational modifications (PTM; such as *S*-nitrosylation and/or nitration, that implicate cysteine and tyrosine residues, respectively) are crucial to convert NO signal into proper physiological responses (Jacques et al., 2013; Kovacs and Lindermayr, 2013). This aspect of NO signaling has been paid much attention in plants in the recent years and led to the identification of hundreds of proteins undergoing such NO-based PTM, and, in a few cases, to a further characterization of the targeted proteins (Kovacs and Lindermayr, 2013). Beyond this direct *modus operandi*, increasing evidence shed light on the intricate relationships between NO and other intracellular signals such as Ca^{2+}, cGMP, phosphatidic acid (PtdOH), or reactive oxygen species (ROS), that trigger, mediate and/or modulate NO signal in response to specific stimuli (Gaupels et al., 2011). A recent review addressed this concern with the example of NO/Ca^{2+} interactions and illustrated some molecular mechanisms through which NO and Ca^{2+} signaling could regulate each other (Jeandroz et al., 2013). Although the underlying mechanisms are less documented, interplays have also been evidenced between NO and the lipid signal PtdOH (Laxalt et al., 2007; Distéfano et al., 2012).

Recently, sphingolipids (SLs), another class of well-known signaling lipids in mammal cells, were ascribed important signaling functions in plants (Berkey and Xiao, 2012; Markham et al., 2013). Numerous examples evidenced crosstalks between SL and NO during (patho)physiological processes in animals (Perrotta et al., 2008). Seminal reports suggest that some interactions could also operate in plants. This mini-review presents our current knowledge of the interactions existing between NO and SL signaling in plants, and put it in perspective with well-documented examples from the animal field.

SPHINGOLIPID SYNTHESIS AND SIGNALING IN PLANTS

The generic term SLs designate both membrane-located complex SL (glycosylceramides, inositol-phosphoceramides, and glycosylinositol-phosphorylceramides in plants) and their metabolic precursors, i.e., long chain bases (LCB) and ceramides (Cer; Pata et al., 2010). They therefore constitute a diverse family of hundreds of molecular entities (Cacas et al., 2012). Adding to this complexity, a subset of LCB and Cer can get phosphorylated by specific LCB and Cer kinases, respectively. Finally the relative amount of the different SL species is not steady, but might undergo fluctuations due to the regulation of SL synthesis, degradation,

and/or phosphorylation/dephosphorylation leading to an over-representation of specific SL (Kihara et al., 2007). Therefore, far away from the early picture of SL being static constitutive entities, the sphingolipidome now emerges as dynamic, possibly modified in response to inside and outside signals and thereafter prompting a range of physiological responses (Markham et al., 2013).

Parallel to the decoding of sphingolipidome, studies conducted during the last decade brought tangible evidences for SL function in signaling networks operating during plant development and responses to environmental cues (Berkey and Xiao, 2012; Markham et al., 2013). Best documented are signaling functions for the precursors of complex SL, i.e., LCB and Cer. For instance LCB and Cer participate in the induction and/or control of plant cell death as illustrated by several studies in which LCB/Cer content was modified by exogenous treatments or the disruption of key genes of SL metabolism (Liang et al., 2003; Lachaud et al., 2010; Saucedo-Garcia et al., 2011; Ternes et al., 2011). The biological relevance of LCB/Cer-triggered cell death has been assumed for plant–pathogen interactions as (i) transient increases of LCB content are observed upon pathogen infection and (ii) pathogen-induced cell death is altered in mutants of SL metabolism (Brodersen et al., 2002; Liang et al., 2003; Peer et al., 2010). Noteworthy complex membrane-located SL also participate in pathogen-triggered cell death (Wang et al., 2008). Furthermore, whereas LCB/Cer promote cell death, phosphorylated LCB (LCB-P) and Cer (Cer-P) prevent cell death (Liang et al., 2003; Shi et al., 2007; Alden et al., 2011). As in mammal cells, the tight control of LCB/LCB-P and Cer/Cer-P equilibrium, and more generally of SL metabolism, is therefore a crucial aspect of plant cell homeostasis keeping it alive or bringing it to death. Whereas the function of Cer/Cer-P has only been investigated in relation with cell death, the role of LCB/LCB-P likely exceeds this limited context. Indeed, mutants of LCB/LCB-P metabolism present altered responses to abiotic stresses unrelated to cell viability. For instance LCB-P have been implicated in a abscisic acid (ABA)-dependent pathway regulating stomatal aperture and drought stress tolerance (Ng et al., 2001; Coursol et al., 2003; Worrall et al., 2008). LCB-P have also been implicated in cold, salt, and oxidative stress responses (Dutilleul et al., 2012; Zhang et al., 2012). These studies have identified several upstream and downstream elements of the SL signaling cascade including Ca^{2+}, heterotrimeric G proteins, ROS, and the MAP kinase AtMPK6. Recent data also suggest that PtdOH signaling can act in a coordinated way with SL (Guo et al., 2011, 2012). Whether these signals are ubiquitous elements of SL signaling is currently unknown.

Less documented in plants are the signaling functions of SL related to their particular location within membrane microdomains (rafts). Rafts are not only enriched in SL and sterols, but also present a particular protein composition (Simon-Plas et al., 2011; Cacas et al., 2012). Indeed, plant membrane rafts are rich in signaling-related proteins (Morel et al., 2006; Lefebvre et al., 2007). Such signaling proteins might not be permanent raft residents but rather temporarily recruited following stimulus perception (Minami et al., 2009; Li et al., 2011). Therefore, rafts emerged as potent signaling platforms and the dynamic modification of membrane structure/composition is probably involved in signal transduction during plant development and response to environmental cues. For instance alterations of membrane integrity via defects in SL composition led to strong developmental phenotypes due to auxin carrier mislocalization (Roudier et al., 2010; Markham et al., 2011). Moreover analysis of the SL and raft abundance and the raft lipid/protein composition during plant acclimation to cold evidenced a close correlation between SL and raft dynamics (Minami et al., 2010). Although the mechanisms underlying the remodeling of rafts is far from being solved, SL have been demonstrated as key components for raft formation in animal membranes (Filippov et al., 2006). As proposed by Cacas et al. (2012), this function of membrane SL is likely conserved in plants, therefore outlining a possible link between SL-based regulation of raft formation and/or structure and SL-triggered signaling events.

INTERPLAYS BETWEEN SL AND NO SIGNALING: SOME LESSONS FROM MAMMAL CELLS

Studies in the animal field initiated in the late 1990s brought to light interconnections between SL and NO signaling in (patho-) physiological situations (reviewed in Huwiler and Pfeilschifter, 2003; Igarashi and Michel, 2008; Perrotta and Clementi, 2010). The models hypothesized from these studies principally implicate Cer and sphingosine 1-P (S1P), the major LCB signal in animal cells (**Figures 1A,B**). First, NO can regulate sphingomyelinases (SMase) that generate the formation of Cer from sphingomyelin (SM), a major membrane SL in mammal cells (**Figure 1A**; Perrotta et al., 2008). Interestingly NO might regulate SMase activities in a different way, depending on the intracellular NO level. On the one hand low physiological concentrations of NO lead to the inhibition of SMases, thereby preventing cell death in a large range of (patho)physiological models by reducing the intracellular Cer concentration (Falcone et al., 2004; Perrotta et al., 2007). On the other hand high levels of NO lead to the increase of Cer concentration, thereby driving cells to apoptosis (Takeda et al., 1999; Pilane and LaBelle, 2004). In this last case (**Figure 1A**), NO promotes (i) the activation of SMases that generate Cer and (ii) represses Cer degradation *via* the inhibition of ceramidase activities (Huwiler et al., 1999; Franzen et al., 2002). How NO up-regulates SMases and down-regulates ceramidases under such conditions is currently unknown. A SMase isoform has been recently identified as *S*-nitrosylated in mouse, thus providing a possible mechanism for SMase regulation by high NO concentration (Kohr et al., 2011). Under low NO concentrations SMase inhibition is likely indirect and involves a cGMP/PKG-dependent pathway, possibly in relation with the regulation of SMase intracellular localization (Falcone et al., 2004; Perrotta and Clementi, 2010).

A second model is illustrated by the regulation of NO formation by the endothelial NO synthase (eNOS) mediated by S1P (**Figure 1B**; Igarashi and Michel, 2008). At least two mechanisms are at play in this process. Firstly the association of eNOS with and its inhibition by caveolin, a transmembrane protein located in the raft-related caveolae microdomains, is reverted by a Ca^{2+}-dependent mechanism mediated by S1P (Igarashi and Michel, 2008). Secondly S1P via its binding to S1P receptors activates a signaling cascade involving AMP-activated protein kinase, Rac1 G protein, PI_3 kinase, and Akt kinase, ending up at eNOS phosphorylation and activation (Levine et al., 2007). Strikingly most

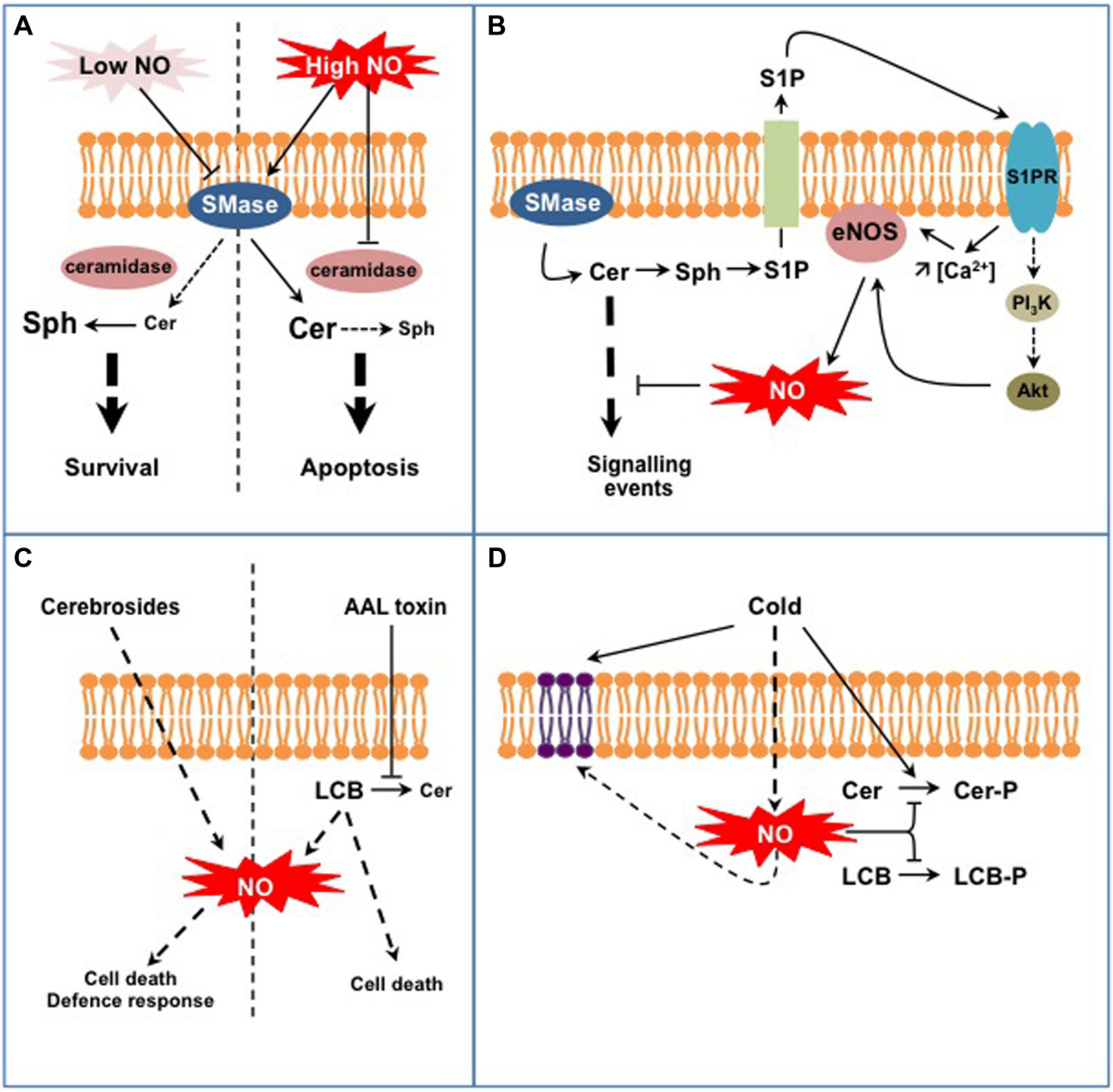

FIGURE 1 | Examples of interplays between SL and NO signaling in animal (A,B) and plant cells (C,D). (A) NO regulates Cer formation from sphingomyelin in a dose-dependent process. Low NO concentrations inhibit sphingomyelinase activity (SMase) leading to low Cer levels that are further degraded to sphingosine (Sph) by ceramidases. High NO concentrations stimulate SMase activities while inhibiting ceramidases, therefore leading to high Cer levels. This differential control of SMases by NO participates in Cer-dependent cell survival or death. **(B)** Cer formation indirectly triggers endothelial NOS (eNOS) activation and NO formation. Sphingosine-1P (S1P) is formed from Cer degradation and subsequent phosphorylation of Sph. S1P is externalized and perceived on the outer cell surface by specific S1P receptors (S1PR). Activated S1PR trigger eNOS activation *via* an increase of cytosolic Ca^{2+} concentration and/or *via* the regulation of the PI_3K/Akt signaling pathway. eNOS-evoked NO eventually down-regulates Cer-activated signaling pathways. **(C)** Complex SL (fungal cerebrosides), SL precursors (LCB), or SL analogs (AAL fungal toxin) act as potent inducers of NO formation. Such NO production might participate in specific aspects of SL-triggered cell death or defense responses. **(D)** Plant exposure to cold triggers the formation of NO that down-regulates the synthesis of phospho-SL (i.e., Cer-P and LCB-P). In addition, NO participates in the modification of membrane SL content resulting from low temperature exposure.

of these proteins are located within caveolae, in close vicinity with eNOS. Such common location also accounts when considering the origin of S1P. As exemplified in TNFα-stimulated HeLa cells, S1P originates from Cer released from SM by a specific SMase isoform (Barsacchi et al., 2003). Cer are subsequently deacylated by ceramidases into sphingosine that gets phosphorylated to S1P. Noteworthy the SMase isoform involved in this model is also located in caveolae together with eNOS (Perrotta and Clementi, 2010). Ultimately eNOS-evoked NO counteracts the apoptotic effects of Cer by inhibiting Cer signaling pathway.

These examples point out the intricate network involving NO and SL in mammal cells. Part of this complexity resides in the diverse isoforms of SMases and ceramidases that undergo different NO-based regulations. Thereby, NO might contribute as an enhancer or a down-regulator of Cer signaling. These examples finally underline that the interplay between NO and SL signaling is not unidirectional, but can also involve bi-directional signaling according to the cellular response examined.

INTERPLAYS BETWEEN SL AND NO SIGNALING IN PLANTS: PROMISES FROM DAWN

At first glance, the models depicted above seem not transposable to plants as most of the molecular actors mentioned are absent from plant cells (e.g., SM, SMases, S1P receptors, eNOS). Nevertheless, several lines of evidence indicate the existence of similar interplays between SL and NO signaling in plants. First, studies have reported the capacity of SL-related molecules to trigger NO synthesis (**Figure 1C**). Wang et al. (2007, 2009) evidenced that treatments with cerebrosides from the fungal pathogen *Fusarium* sp IFB-121 induce NO formation in *Taxus yunnanensis* and *Artemisia annua*. Cerebrosides are complex membrane SL widely found in soilborne fungi and are considered as pathogen-associated molecular patterns (PAMP; Umemura et al., 2004). In this context, cerebroside-evoked NO triggers the synthesis of secondary metabolites, i.e., taxol and artemisin (Wang et al., 2007, 2009). In addition to SL-related elicitors, some pathogenic fungi produce toxins structurally analogous to LCB, such as AAL toxin from *Alternaria alternata* f.sp. *lycopersic*i or fumonisin B1 (FB1) from *Fusarium moniliforme*. Although the formation of NO in response to these toxins has not been directly evidenced, AAL-triggered cell death was blocked by an inhibitor of mammalian NOS suggesting that NO was required for AAL response (Gechev et al., 2004). Being LCB analogs, AAL and FB1 toxins block Cer synthesis and provoke free LCB accumulation (Abbas et al., 1994). Interestingly, Da Silva et al. (2011) recently showed that exogenous treatments with LCB triggered NO formation in tobacco cells. Nevertheless the biological outcome of LCB-stimulated NO production remains obscure as NO was not required for LCB-induced cell death. Although seminal, these studies require further investigations to establish the biological relevance of SL-triggered NO formation. For instance, one has to establish if specific SL structural features are required to trigger NO production, as reported for H_2O_2 synthesis (Shi et al., 2007). As plants lack *bona fide* NOS, the source of SL-evoked NO should be hunted, together with the mechanisms underlying its activation by SL. Finally it is noteworthy that the data available rely on exogenous treatment of plant material with SL-related molecules. One has therefore to examine if and how endogenous modifications of SL homeostasis might induce NO production.

Conversely to the regulation of NO formation by SL, recent data indicate that NO regulates specific aspects of SL metabolism in plants (**Figure 1D**). In particular it may participate in the fine-tuning of the equilibrium between LCB/Cer and LCB-P/Cer-P. This was evidenced for *Arabidopsis* response to cold where two phosphorylated SL species (i.e., the LCB phytosphingosine-phosphate and a putative Cer-P) are rapidly and transiently formed (Cantrel et al., 2011; Dutilleul et al., 2012). In this context cold-evoked NO functions as a negative regulator of phospho-SL formation (Cantrel et al., 2011). How NO regulates phospho-SL formation during cold-stress response remains unclear. Firstly NO could impact the activity of kinases or phosphatases metabolizing LCB-P and Cer-P. For instance sphingosine kinase 1 (SPHK1) is regulated by NO in human endothelial cells (Schwalm et al., 2010). So far only S1P lyase, which catalyses the degradation of LCB-P, has been identified as a target for NO-based PTM and the biological significance of this modification remains to be established (Zhan and Desiderio, 2009). The regulation of LCB-metabolizing enzymes has been poorly studied in plants and further investigations are therefore required to decipher if NO can directly regulate these enzymes. Secondly NO might modify the availability of LCB/Cer kinase substrates. Supporting this possibility, Guillas et al. (2013) evidenced that an *Arabidopsis* mutant line over-expressing a non-symbiotic hemoglobin, and thereby exhibiting low NO levels, over-accumulates phytosphingosine. The levels of phytosphingosine were further increased after cold exposure and might afford for the highest rate of phytosphingosine-P formation observed in this mutant (Cantrel et al., 2011). Interestingly, the analysis revealed another facet of the SL response affected in this mutant. Indeed, whereas the overall amount of LCB was strongly lowered by cold exposure in WT plants, it was drastically increased in the mutant line (Guillas et al., 2013). These data therefore suggest that NO might participate in the regulation of more complex sphingolipidome modifications associated with cold response.

As for SL-triggered NO formation, the example presented above questions about the ubiquity of SL–NO interplay in diverse physiological contexts and the underlying mechanisms at work. Although direct connections have not been established yet, it is likely that SL–NO crosstalks participate in ABA signaling (Zhang et al., 2009; Guo and Wang, 2012). In this framework PtdOH metabolism and signaling could be crucial to interlink SL and NO signaling. Indeed ABA activates phospholipase Dα1 (PLDα1) to synthesize PtdOH and thereby triggers NO formation (Zhang et al., 2009; Uraji et al., 2012). Strikingly PLDα1 is also a target for LCB-P that stimulate PtdOH synthesis (Guo and Wang, 2012). This apparent simplicity turns to complexity when considering that (i) PtdOH generated by PLDα1 interacts with and further stimulates the LCB kinase SPHK1 (Guo et al., 2012) and (ii) that a ABA-triggered NO production is also required for the activation of Phospholipase Dδ and PtdOH synthesis (Distéfano et al., 2012). In this intricate signaling network, further investigations should now examine the consequences of alterations of NO or SL signaling on each other to clearly establish possible direct NO–SL crosstalks. The interaction between *Arabidopsis* and the phytopathogenic bacteria *Pseudomonas syringae* is another context where NO–SL interactions are likely. On the one hand studies carried out on this pathosystem led to the pioneering demonstration of NO as a signal in plants (Delledonne et al., 1998). On the other hand it provided the first example of dynamic changes of LCB level triggered by biotic stress in plants (Peer et al., 2010). Although the interplay of NO and LCB has not been addressed yet in this system, it opens the possibility that NO regulates LCB metabolism as suggested above for cold stress, and/or that LCB trigger NO production as reported for ROS (Peer et al., 2011). Besides interacting within signaling

networks or interfering with each other metabolism, NO and SL might interfere at the level of protein trafficking toward membranes. In the case of auxin bioactivity, defects in either SL or NO metabolism lead to misaddressing or degradation of the auxin efflux transporter PIN1 and thereby to altered development of root system (Fernández-Marcos et al., 2011; Markham et al., 2011; Yang et al., 2013). Due to the recent involvement of NO in vesicle trafficking in roots (Lombardo and Lamattina, 2012), further analysis of its interplay with SL in this context might shed light on unexplored roles for NO in plant cell biology.

CONCLUSION

Increasing evidence plead for functional interplays between NO and lipid signaling and indirectly bring to forestage the role of biological membranes in NO biology. As exemplified in mammals and plants, SL signals generated by the catabolism or as intermediates of the synthesis of complex membrane SL, constitute new elements of the NO signaling network in a variety of physiological processes. The rising interest for SL and NO signaling in plants will undoubtedly provide soon new examples of this interplay. Future investigations should help unravel the mechanisms underlying such NO–SL signaling crosstalks. In particular a direct regulation of enzymes of the NO and SL pathway by SL and NO, respectively should be evaluated. As observed in mammals, this might include modulation of activity but also regulation of protein targeting. Finally it is likely that NO, which is liposoluble, does not only interplay with SL signaling within the cytosol, but also within the biological membranes. As it might deeply affect the activity and/or targeting of membrane-located proteins and the overall membrane structure, attention should now be paid to NO signaling within the lipid phase.

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Nitric oxide implication in the control of seed dormancy and germination

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Germination ability is regulated by a combination of environmental and endogenous signals with both synergistic and antagonistic effects. Nitric oxide (NO) is a potent dormancy-releasing agent in many species, including *Arabidopsis*, and has been suggested to behave as an endogenous regulator of this physiological blockage. Distinct reports have also highlighted a positive impact of NO on seed germination under sub-optimal conditions. However, its molecular mode of action in the context of seed biology remains poorly documented. This review aims to focus on the implications of this radical in the control of seed dormancy and germination. The consequences of NO chemistry on the investigations on both its signaling and its targets in seeds are discussed. NO-dependent protein post-translational modifications are proposed as a key mechanism underlying NO signaling during early seed germination.

Keywords: abscisic acid, dormancy, germination, nitric oxide, seed, vigor

INTRODUCTION

Survival of plant species mainly relies on the sexual reproduction which gives rise to new populations. During this process, the adult angiosperm plants produce flowers which upon fertilization give rise to seeds, the main unit of dispersal of flowering plants. In the plant life cycle, the seed and seedling stages are key developmental stages conditioning the final yield of crops. Indeed, seed dormancy, viability, and germination vigor are among the main concerns for agricultural productivity. High vigor seed lots display a low dormancy and lead to seedlings able to withstand extreme stress conditions. If not completely released, dormancy will negatively influence seed germination, which is detrimental to crop yield. However, from an agronomical point of view, lack of dormancy is not a desirable trait as it may lead to pre-harvest sprouting (Bewley and Black, 1994). Therefore, the management of this trait is of fundamental concern for the seed industry and agriculture performance. Thus, investigation of seed quality, toward a better understanding of dormancy, germination and longevity, is of paramount agronomical importance. All these seed features are complex traits controlled by a large number of genes, which are affected by both developmental and environmental factors.

Numerous distinct nitrogen-containing compounds have been shown to positively influence seed germination especially by releasing seed dormancy and improving seed vigor in a wide range of species (Bethke et al., 2007b). These concentration-dependent effects could allow the sensing of the presence of these essential resources in the direct environment. The possibility that all these molecules could act in a similar way prompted plant biologists to look for a possible common nitrogen-containing intermediate and pinpointed nitric oxide (NO) as a possible candidate. Indeed, since its discovery, this radical has progressively emerged as an ubiquitous molecule in both animal and plant signaling networks (Baudouin, 2011). Increasing reports highlight its large implication in diverse signaling pathways regulating growth and developmental processes all along the plant life cycle. A key role for NO was further demonstrated in plant response to abiotic and biotic stresses. Instead of describing in details all these roles that have already been extensively discussed in recent reviews (Besson-Bard et al., 2008; Wilson et al., 2008; Moreau et al., 2010; Baudouin, 2011), we will focus on the implications of NO in the control of seed dormancy and germination with a particular emphasis on the experiments carried out on the model Angiosperm plant *Arabidopsis thaliana*. The present review also aims to provide outlooks for future investigation in this field.

DEFINITION AND GENERAL OVERVIEW ON SEED DORMANCY AND GERMINATION

SEED DORMANCY

Under natural conditions, an appropriate timing of seed germination is determinant to ensure optimal growth conditions for the young seedlings and guarantee the survival of the species (Bewley, 1997). Seed dormancy is one of the mechanisms contributing to this spatio-temporal adjustment and is defined as a block to the completion of germination of an intact viable seed placed under (temporary) favorable conditions in an otherwise unfavorable

season (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Graeber et al., 2012). It may be due to certain properties of the seed coat, mobilization of reserve components, hormone levels, or the joint action of several of these factors (Koornneef et al., 2002). Thus, dormancy is determined by genetic factors but it can also be substantially modulated by environmental parameters (Graeber et al., 2012). Indeed, the alleviation of this blockage can be conditioned by several distinct environmental (temperature, humidity, light, nutrient concentration. . .) or physical (testa rupture. . .) factors. The exact conditions required for dormancy release and subsequent germination depend on the species and thus contribute to the adequacy of the plant to its environment by delaying germination until the seed meets appropriate conditions for its development. In addition, the depth of primary dormancy in mature seeds can depend on the conditions under which the mother plant was exposed such as temperature or availability of mineral elements (such as nitrate) in the soil (Alboresi et al., 2005; Kendall et al., 2011). Thus, seeds have developed a complex control of the depth of dormancy integrating diverse spatio-temporal parameters allowing a dynamic definition of the minimal requirements for germination. In addition, when a non-dormant seed encounters inappropriate conditions for germination, it can enter into a so-called secondary dormancy. Overall, these mechanisms contribute to the sensing of environmental conditions and can lead to dormancy cycling under natural conditions (Footitt et al., 2011).

Abscisic acid (ABA) is considered as the pivotal hormone responsible for the induction and maintenance of seed dormancy (Nambara et al., 2010). ABA is accumulated during seed maturation reaching high levels in dry seeds. Dry dormant seeds were found to contain higher amounts of ABA than dry after-ripened non-dormant seeds (Ali-Rachedi et al., 2004). Upon imbibition, a significant decrease in ABA content was observed in both dormant and non-dormant seeds (Ali-Rachedi et al., 2004). However, after 3 days of imbibition a significant up-accumulation of ABA was detected in dormant seeds only. Exposition of dormant seeds to common dormancy-releasing treatments such as cold-stratification or exogenous nitrate supply leaded to ABA levels similar to non-dormant seeds and prevented the increase in ABA observed when dormancy is maintained (Ali-Rachedi et al., 2004). Reactive oxygen species (ROS) and NO counteract the positive effect of ABA on seed dormancy maintenance. Exogenous application of fluridone (an inhibitor of ABA synthesis) also efficiently released seed dormancy by reducing ABA levels highlighting the requirement for de novo ABA synthesis for the maintenance of this blockage and the existence of a dynamic equilibrium between ABA synthesis and catabolism during seed imbibition (Ali-Rachedi et al., 2004). In addition, recent experiments demonstrated that two independent dormancy-releasing treatments led to similar proteome adjustments supporting the occurrence of shared molecular mechanisms underpinning seed dormancy release (Arc et al., 2012). Furthermore, recent data emphasize the importance of redox control of seed proteome in dormancy release (Marx et al., 2003; Bykova et al., 2011a,b). Thus, ROS and NO appear as good candidates, acting synergistically to release dormancy, putatively acting upstream of ABA.

SEED GERMINATION

Seed germination is temporally defined as the sequence of molecular and physiological events initiated upon imbibition of non-dormant seed and leading to the radicle protrusion through the seed external envelopes (testa and endosperm) that marks the end of germination *sensu stricto* (Bewley, 1997). Seed germination constitutes a pivotal physiological transition and is associated with a strong modification of the transcriptome (∼one-third of the genome) and metabolism over a short time period (around 36–48 h for non-dormant *Arabidopsis* seeds) relatively to the plant life cycle. During this process, the initially quiescent dry seed successively go through three major steps of water uptake (Bewley, 1997; Weitbrecht et al., 2011). The first step consists in a rapid imbibition of the initially quiescent seeds that lead to the progressive resumption of metabolic activity, gene expression (transcription), protein synthesis and processing and DNA repair (Weitbrecht et al., 2011). The recapitulation of the metabolic activity mainly depends on the stored proteins and metabolites. The importance of the compounds accumulated in the seeds during the maturation was further highlighted by the finding that stored mRNAs and proteins are sufficient for germination *sensu stricto* (Rajjou et al., 2004; Sano et al., 2012). *De novo* protein synthesis from the stored mRNAs occurs during the very early step of germination. During this period, the proteins translated are similar to those accumulated during the late maturation and already abundant in seeds reflecting an early recapitulation of the corresponding gene expression program during early germination (Rajjou et al., 2006, 2012). During the second step of water uptake, the water content only slightly increases while important metabolic changes take place inside the seeds. A significant shift is observed during this step from maturation to germination program of development that includes the preparation for seedling establishment (Lopez-Molina et al., 2002; Nonogaki et al., 2007). This two steps time course is consistent with a model proposing that recapitulation of the late maturation program occurs during early germination up to an ABA-dependent developmental checkpoint after which the seed can either activate its germination program or maintain a dormant state notably depending on the sensing of environmental conditions during early imbibition (Lopez-Molina et al., 2002; Rajjou et al., 2012). During this period, seeds maintain their desiccation tolerance. At the end of this second step, if the "decision" to pursue toward germination is taken, the growth potential of the embryo progressively overcome the mechanical constraints imposed by the surrounding layers leading to the successive rupture of the testa and the endosperm (Nonogaki, 2006; Bentsink and Koornneef, 2008). The protrusion of the radicle through the seed coat is thus achieved as a result of important cell elongation without any cell division (Sliwinska et al., 2009) and occurs concomitantly with an important resumption of water uptake. The ABA/gibberellins (GAs) balance coordinate this last step with a decrease in ABA leading to the progressive release its inhibitory effect on endosperm rupture while an important increase in bioactive GAs levels both enhanced the growth potential of the embryo and induced hydrolytic enzymes that weaken the barrier tissues (Bewley and Black, 1994; Muller et al., 2006; Finkelstein et al., 2008).

GERMINATION VIGOR

If the seed encounters suitable conditions for germination during its life, it may, if still viable, allow the young seedling establishment. But as a consequence of aging, the seed germination vigor can be severely affected. In other words, the capacity of a seed lot to germinate rapidly, uniformly and in a wide range of environmental conditions can be impaired or destroyed. As the seed germination process mainly relies on stored mRNA and proteins (Rajjou et al., 2004), damages at the DNA level can result in an aborted development of the seedling. Thus, cellular repair mechanisms especially at the DNA level but also for certain protein post-translational modifications (PTMs) play an essential role in seed vigor (Rajjou et al., 2012). Due to seed high vulnerability to injury, abiotic, and biotic stresses during imbibition, germination is considered as the most critical phase of the plant life cycle. The level of reactive oxygen and nitrogen species (respectively ROS and RNS), influenced by the storage and environmental conditions will determine a balance between the required signaling events and the detrimental oxidative damages (Bailly et al., 2008; Rajjou et al., 2008, 2012; Arc et al., 2011).

NITROGEN OXIDES IMPLICATION IN THE CONTROL OF SEED DORMANCY AND GERMINATION

NITRATE AND NITRITE AVAILABILITY: DETERMINANT FACTORS FOR SEED DORMANCY RELEASE AND SUBSEQUENT GERMINATION

Nitrate (NO_3^-) is considered as a major nitrogen source for most plant species. Nitrate reduction into nitrite (NO_2^-) is catalyzed by nitrate reductase (NR) that produces nitrogen-containing metabolites, such as amino acids and NO. Apart from being an essential nutrient, nitrate is also considered as a signaling molecule involved in both plant metabolism regulation and developmental processes (Krouk et al., 2010). In particular, nitrate has been shown to promote seed dormancy release and subsequent germination in numerous plant species (Bewley and Black, 1994). Most of the first experiments mainly investigated the effect of nitrate on these physiological processes although the principal product of its assimilation, nitrite can also alleviate seed dormancy (Bethke et al., 2006a).

Exogenous treatments with nitrates were shown to promote seed germination in *Arabidopsis* by reducing the light requirement (Hilhorst and Karssen, 1988; Batak et al., 2002). The enhancement of germination mediated by light absorbed by phytochrome-A operates via the very-low-fluence response (VLFR; Botto et al., 1996). Thus, nitrate could stimulate the accumulation of cGMP, which then promotes some phytochrome responses (Ludidi and Gehring, 2003). Moreover, a positive correlation between endogenous or applied nitrate levels and germination response to ethylene or GAs was reported for *Chenopodium album* seeds (Saini et al., 1985). In *Arabidopsis*, high nitrate feeding of mother plants is associated with higher nitrate content and lower dormancy of the seed progeny (Alboresi et al., 2005). This result suggests a negative correlation between nitrate levels in dry mature seeds and the depth of dormancy. In addition, mutation in the nitrate transporter NRT1.1/CHL1 resulted in lower sensitivity to exogenous nitrate indicating that this protein may be required for nitrate uptake by the seed (Alboresi et al., 2005). Moreover, mutants in the seed specific nitrate transporter AtNRT2.7, involved in nitrate loading into the vacuole during seed maturation, displayed reduced nitrate content and slightly increased dormancy (Chopin et al., 2007). Overall, nitrate availability in seeds appears as an important determinant of seed dormancy.

The reduced dormancy of NR deficient seeds, impaired in nitrate assimilation, along with the finding that glutamine, another nitrogen source did not affect seed germination suggest that the effect of nitrate is unrelated to plant nutrition (Alboresi et al., 2005). As stated in the previous part, exogenous nitrate application was proved to negatively affect ABA content during *Arabidopsis* seed imbibition (Ali-Rachedi et al., 2004). In addition, controlled nitrate supply to the mother plants led to ABA contents negatively correlated to the endogenous nitrate concentration in dry mature seeds (Matakiadis et al., 2009). Accordingly, it has recently been demonstrated that the gene expression of the ABA catabolic enzyme, CYP707A2, was positively regulated by both endogenous and exogenous nitrate (Matakiadis et al., 2009). Thus, the positive effect of nitrate on dormancy alleviation is presumably mediated by affecting ABA metabolism.

NITRIC OXIDE, THE KEY SIGNALING ELEMENT MEDIATING NITRATE RESPONSE IN SEEDS?

Nitric oxide is a gaseous diatomic free radical detected at low levels in the atmosphere. It is also present in the soils at a concentration depending on the micro-biotic environment (Simontacchi et al., 2007). Moreover, nitrogen fertilization was shown to increase NO release from the soils and proposed to account for the fitness of nitrogen-fertilized plants (Lamattina et al., 2003). NO was shown to efficiently break the dormancy and / or promote germination of several orthodox seeds (Beligni and Lamattina, 2000; Bethke et al., 2004b; Sarath et al., 2006; Liu et al., 2007; Gniazdowska et al., 2010a) including in *Arabidopsis thaliana* (Bethke et al., 2006b).

Nitric oxide: a key mediator of seed dormancy release

Recent data disclosed that the improvement of dormant-seeds germination provided by exogenous treatments with various nitrogenous molecules, including nitrate, and nitrite, most presumably occurs through NO production (Bethke et al., 2004b, 2006a). Accordingly, the NO content in homogenates from 24 h-imbibed soybean and sorghum embryonic axes, detected by electron paramagnetic resonance (EPR)-spin trapping, increased with increasing nitrate supply during seed imbibition (Caro and Puntarulo, 1999; Simontacchi et al., 2004). This result pinpoints exogenous nitrate concentration during seed imbibition as a key determinant of NO release.

Indeed, NO is well known to release seed dormancy in numerous species (Bethke et al., 2007b). For instance, pharmacological approaches demonstrated that most known NO donors promoted dormancy alleviation and subsequent germination while NO scavengers favored dormancy maintenance and counteracted the positive effect of NO donors (Bethke et al., 2007a). In addition, it has been shown that NO may alleviate dormancy of apple embryos *via* a transient accumulation of ROS, leading to enhanced ethylene emission as required to terminate germination *sensu stricto* (Gniazdowska et al., 2007, 2010a,b). NO also proved efficient to reverse blue light inhibition of dormant wheat seed germination,

presumably acting interdependently with methyl-jasmonates in controlling reduction of ABA (Jacobsen et al., 2013).

In tomato seeds, the NO scavenger, carboxy-2-phenyl-4,4,5-tetramethylimidazole-1-oxyl 3-oxide (cPTIO), was shown to prevent germination stimulation by fluridone, an ABA synthesis inhibitor (Piterkova et al., 2012). On the contrary, exogenous sodium nitroprusside (SNP), commonly used as NO donor, enhanced the positive effect of norfluorazon, another ABA synthesis inhibitor, on dormancy release of *Arabidopsis* C24 seeds (Bethke et al., 2006b). Moreover, SNP was shown to reduce seed sensitivity to exogenous ABA (Bethke et al., 2006b). Taken together, these results suggest that NO can decrease ABA sensitivity. A possible effect of NO on ABA catabolism was consequently investigated. Seed treatment with NO donor enhanced CYP707A2 transcript and protein accumulation while the NO scavenger c-PTIO reduced CYP707A2 expression and reversed the NO donor effect (Liu et al., 2009). Thus, as for nitrate, NO was found to enhanced *CYP707A2* gene expression (Liu et al., 2009). These results consequently reinforce the assumption that nitrate does not affect seed dormancy on its own but rather act through NO biosynthesis.

A rapid accumulation of NO, possibly in the endosperm layer, during the first stage of *Arabidopsis* seed imbibition is required for rapid ABA catabolism and breaking of dormancy (Liu et al., 2009). A similar NO accumulation during imbibition was also observed in germinating seeds from other species (Simontacchi et al., 2007). Recently, NO was suggested to act upstream of GAs in a signaling pathway leading to vacuolation of protein storage vacuoles in aleurone cells, a process inhibited by ABA (Bethke et al., 2007a). However, the growth of isolated embryos was unaffected by NO donor or scavengers. Thus, the endosperm layer, proposed as the primary determinant of seed dormancy in *Arabidopsis*, was proved to perceive and respond to NO, and suggested as its main site of synthesis and action in seeds (Bethke et al., 2007a). Apart from its effect on the hormonal balance, it has been speculated that NO might accelerate the flux towards the pentose phosphate pathway (PPP) by indirectly increasing the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH; Hendricks and Taylorson, 1974; Bethke et al., 2007b). Interestingly, the oxidation of NADPH by *S*-nitrosoglutathione (GSNO) in the presence of thioredoxin reductase and thioredoxin was demonstrated, releasing glutathione (GSH) and NO (Nikitovic and Holmgren, 1996). In addition, the involvement of the hemoglobin/NO in the oxidation of NADPH has been proposed (Igamberdiev and Hill, 2004). An increase in glucose catabolism via PPP could in turn promote dormancy release (Roberts and Smith, 1977).

As a conclusion, NO is a likely player of a signaling pathway that promotes loss of dormancy and has been suggested to behave as an endogenous regulator of this process. However, the direct targets of NO in seeds remain unclear. Nonetheless, some consequences of NO accumulation on seed metabolism have been highlighted and pinpoint an implication in the regulation of ABA metabolism.

Reactive oxygen species and no crosstalk in the control of seed dormancy and germination

In parallel to NO, ROS have emerged as key players in the control of seed dormancy and germination (Bailly, 2004; Bailly et al., 2008). In cells, ROS can be generated by specific enzymatic reactions or as by-products of the metabolism. Depending on their concentration, ROS may have positive signaling effects including the promotion of dormancy release and germination or detrimental consequences (Liu et al., 2010; Leymarie et al., 2012). Accordingly, it has been proposed that the amount of ROS generated upon seed imbibition should fall within a defined "oxidative window" for germination to occur (Bailly et al., 2008). Below this window, ROS levels would be too low to promote dormancy alleviation while above, oxidative damages would be predominant.

Recently, it has been proposed that ROS might coordinate the reduction of ABA-imposed dormancy with the onset of GA-stimulated germination (Liu et al., 2010). More precisely, exogenous hydrogen peroxide (H_2O_2) was shown to enhance ABA catabolism and GA biosynthesis during seed imbibition. As NO scavenger efficiently reversed H_2O_2-mediated induction of *CYP707A* genes but had no effect on the stimulation of GA biosynthesis, NO was proposed to act downstream of H_2O_2 in enhancing ABA catabolism. *In vivo*, both H_2O_2 and NO appeared to accumulate rapidly and concomitantly upon imbibition and to precede the induction of ABA catabolism/GA biosynthesis (Liu et al., 2010).

In stomatal guard cells, one of the well-established signaling pathway for ABA-induced stomatal closure involve the successive accumulation of ROS and NO, acting as secondary messengers of ABA signal (Neill et al., 2008; Simontacchi et al., 2013). Even though similar actors are present in seeds, the picture is obviously quite different as both ROS and NO counteract ABA-inhibition of seed germination. This clear distinction highlights the specificity of seed physiology (**Figure 1**). The exact interplay between reactive nitrogen and oxygen species is always difficult to interpret due to the non-enzymatic reactions susceptible to occur and the molecular consequences they might have.

Nitric oxide and germination vigor

Nitric oxide is well known to play a dual role in stress responses in plants (Corpas et al., 2011). In particular, NO can directly scavenge certain ROS such as superoxide anions and lipid-derived radicals and was shown to stimulate antioxidant enzymes thereby limiting oxidative damages. However, uncontrolled NO accumulation referred to as nitrosative stress can have detrimental consequences.

In seeds, pharmacological experiments highlighted that NO did not significantly influence the germination of non-dormant (fully after-ripened) *Arabidopsis* seeds under optimal conditions (Bethke et al., 2006a). However, in rice seed, NO was proved to enhance germination by stimulating the transcription of the plasma membrane intrinsic protein (PIP) genes encoding water channels (Liu et al., 2007). In addition, several studies suggested that NO could participate in the tolerance to abiotic stresses during seed germination (Sirova et al., 2011). In particular, NO was demonstrated to delay programmed cell death of barley aleurone cells by promoting the activity of antioxidant enzymes (Beligni et al., 2002). In addition, SNP, commonly used as NO-donor, was shown to alleviate heavy metal stress during seed germination of wheat (Hu et al., 2007), lupin (Kopyra and Gwóźdź, 2003) and rice (Péres da Rocha Oliveiros Marciano et al., 2010). Seed pre-incubation (seed priming) with SNP was also proved to increase salt stress tolerance in wheat (Duan et al., 2007; Zheng et al., 2009). Finally, two recent papers on *Arabidopsis* reported an enhanced sensitivity of

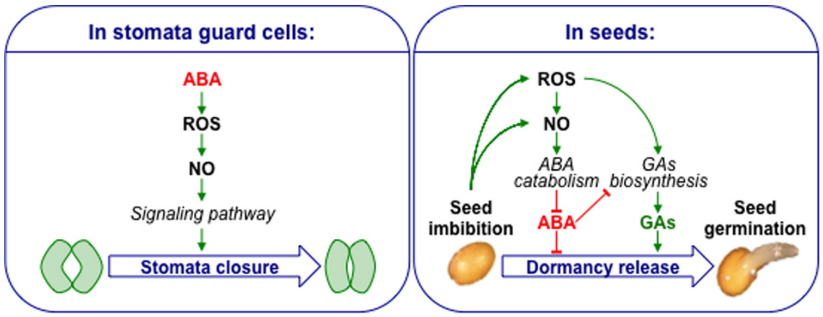

FIGURE 1 | Contrasting models showing ROS, NO, and ABA crosstalk in stomata guard cells and seeds. ABA increases ROS and NO level in guard cells leading to ABA-dependent stomatal closure. Seed imbibition leads to ROS and NO accumulation. ROS up-regulate ABA catabolism through NO, and also GA biosynthesis. A high concentration of ABA also inhibits GA biosynthesis, but a balance of these two hormones jointly controls seed dormancy and germination.

mutants with reduced NO accumulation (*atnoa* and *nia1nia2*) to salt and osmotic stress (Zhao et al., 2007; Lozano-Juste and Leon, 2010). In the few cases where it was examined, stress tolerance was associated to increased antioxidant activity. NO could therefore play a key role in germination vigor that could result from its crosstalk with ROS. NO and superoxide rapidly combine to form peroxynitrite ($ONOO^-$), a selective oxidant able to reacts with most biological molecules. Peroxynitrite modifies protein tyrosine to create nitrotyrosines, leaving a footprint detectable *in vivo* (Vandelle and Delledonne, 2011). However, up to now, only indirect evidences support this assumption in *Arabidopsis* seeds and none investigated the underlying mechanisms associated to the increased tolerance observed. Overall, NO could play a pivotal role in the sensing of environmental conditions appropriate for seed germination.

CONSEQUENCES OF NITRIC OXIDE CHEMISTRY ON THE INVESTIGATION ON ITS SIGNALING IN SEEDS

SPECIFICITIES OF NITRIC OXIDE CHEMISTRY AND SIGNALING

Nitric oxide ($NO^\bullet$) is an uncharged, gaseous and lipophilic free radical that can readily diffuses across biological membranes. Thus, NO can interact with numerous distinct molecules in plant cells and therefore acts as a signaling element. Free $NO^\bullet$ is a transient compound displaying a high reactivity toward other free radicals (e.g., superoxide anion) and transition metal ions (e.g., iron; Wink and Mitchell, 1998). Thus, upon production, released NO can adjust to the cellular redox environment leading to the formation of diverse biologically active compounds collectively referred to as reactive nitrogen species (RNS). RNS include nitrosonium (NO^+) and the nitroxyl anion (NO^-), respectively resulting from a gain or loss of one electron by NO and peroxynitrite ($ONOO^-$) product of the reaction of NO with superoxide anion radical (O_2^-; Stamler et al., 1992b). Oxidation reactions in the presence of molecular oxygen (O_2) can also lead to nitrogen dioxide (NO_2), nitrous anhydride (N_2O_3), NO_2^- and NO_3^- generation. All these molecules differ in reactivity toward the range of NO biological targets. Their differential production can thus orient and/or alter the message mediated by NO. Under physiologic conditions, a strict control of NO content is required to maintain proper cellular functions. High accuracy in signaling events can only be achieved through a tight spatio-temporal control of the intracellular levels of the messengers. Therefore, the balance between NO production and elimination (conversion or storage) is of major importance in determining the biological effects of this radical (Besson-Bard et al., 2008; Moreau et al., 2010; Baudouin, 2011). As for ROS, the chemical reactivity of NO (and associated RNS), make it a particular signal element which can readily interact with a wide range of targets (e.g., proteins, lipids) rather than interact with "dedicated" receptors (Kalyanaraman, 2004; Besson-Bard et al., 2008). The signal mediated by NO can belongs to transduction pathways or be associated with nitrosative stress depending on the biological environment.

NITRIC OXIDE SYNTHESIS AND HOMEOSTASIS IN PLANT SEEDS

Distinct pathways have been proposed to account for NO generation in plant cells (Reviewed in Gupta et al., 2011a). However, the reactions and enzymes involved are still a matter of debate and the relative contribution of these NO biosynthesis pathways remains unclear in seeds (Reviewed in Arc et al., 2013). For instance, NR-catalyzed reduction of nitrite into NO in the cytosol is presumably the most documented reaction but its relevance in seeds is controversial. Instead, nitrite reduction was suggested to occur either via non-enzymatic reactions especially within the apoplasm possibly next to the endosperm layer (Bethke et al., 2004a) or in hypoxic mitochondria (Igamberdiev et al., 2010; Gupta and Igamberdiev, 2011). Alternatively, NO synthesis could result from oxidative reactions from hydroxylamine, polyamines or L-arginine (L-Arg) pathways. NO can also be "stored" through its interaction with diverse molecules. Indeed, NO can react with reduced GSH or thiol groups leading to the reversible formation of *S*-nitrosothiols (e.g., GSNO, *S*-nitrosylated proteins). GSNO was suggested to constitute a storage and transport form for NO, even in seeds (Sakamoto et al., 2002; Catusse et al., 2008).

NITRIC OXIDE DETOXIFICATION BY NON-SYMBIOTIC HEMOGLOBINS

Hemoglobins are well known in the animal kingdom for their role as oxygen carrier. In plants, non-symbiotic hemoglobins (nsHb) are divided into two main classes with distinct properties. Class 2 nsHb are the only proteins with an affinity for oxygen fitting with a direct role in oxygen storage and supply (Spyrakis et al., 2011;

Vigeolas et al., 2011). Contrarily, the very high affinity for oxygen (in the order of 1–2 nM) displayed by class 1 nsHb is not compatible with such function (Dordas, 2009; Gupta et al., 2011b; Hill, 2012). The plant nsHb1 can act as NADPH-dependent dioxygenase metabolizing NO into nitrate (Igamberdiev and Hill, 2004; Perazzolli et al., 2004). Under hypoxia, NO can be generated from nitrite by deoxyhemeproteins within the mitochondria. Then, nsHb1 and NR can allow the NADPH-dependant re-oxidation of NO into nitrite in the cytosol. As NO can reversibly inhibits cytochrome c oxidase, the reaction between NO and nsHb1 is part of a dynamic equilibrium allowing a tight adjustment of the cellular energy and redox state to oxygen availability (Hebelstrup et al., 2007). These reactions constitute the so-called hemoglobin-NO cycle (Igamberdiev et al., 2010). Furthermore, nsHb1 protein also participates in NO scavenging and therefore NO homeostasis. Accordingly, modulation of nsHb1 expression in plants was shown to directly impact NO levels at distinct developmental stages including seeds (Hebelstrup and Jensen, 2008; Thiel et al., 2011) and in diverse environmental conditions (Dordas, 2009; Cantrel et al., 2011). Thus, despite putative other functions, like CO binding (Hill, 2012), the use of transgenic lines with altered AHb1 expression proved to be a valuable tool to highlight NO implication in physiological processes and stress tolerance.

The over-expression of *Arabidopsis* nsHb1, AHb1 (also named GLB1 or AtHb1 in other studies; At2g16060) in seeds resulted in a pre-adaptation to stress with the repression of energy consuming pathways, modulation of hormone metabolisms (ABA, SA, auxin, ethylene. . .) and reduced NO emission under transient hypoxia (Thiel et al., 2011). Overall, this leaded to a more efficient allocation of energy resources in seeds resulting in higher weight of mature transgenic seeds (Thiel et al., 2011). Thus, this study highlighted an impact of AHb1 over-expression on the nitrosative stress induced by hypoxia and possibly on NO mediated signaling during seed maturation. However, in dry mature wild-type (WT) seeds, neither nsHb1 protein nor the corresponding mRNA has been detected so far, instead both accumulated during seed imbibition suggesting a crosstalk between nsHbs1 and NO in the germination process (Duff et al., 1998; Ross et al., 2001; Hebelstrup et al., 2007; Matilla and Rodriguez-Gacio Mdel, 2013). Indeed, the NO dioxygenase activity of nsHb1 may also have a significant impact on seed physiology. Importantly, NO accumulation upon water uptake seems to precede nsHb1 induction (Hebelstrup et al., 2007; Liu et al., 2009; *Arabidopsis* EFP-browser dataset, Winter et al., 2007). The shift between the induction of NO release and nsHb1 accumulation could delimit a short time window during which NO-mediates its effect on ABA catabolism thereby allowing dormancy release before the re-establishment of NO homeostasis by nsHb1 as required to avoid nitrosative stress.

Previous studies relying on modulation of nsHb1 expression in seeds mainly focused on seed maturation (Thiel et al., 2011; Vigeolas et al., 2011). Yet, to date, the link between the NO-related AHb1 function and physiology of seed germination (dormancy, germination vigor, longevity) has never been addressed.

DETECTION AND STUDY OF NITRIC OXIDE IN SEEDS

The investigations on the mode of action of NO in plant cells still suffer from several technical limitations. Indeed, the improvement of NO detection and quantification, pharmacological approaches and biochemical assay for the analysis of NO-induced PTMs are still required.

Pharmacological experiments

Most of the known implications of NO in plant physiology were first highlighted through pharmacological experiments employing NO donors and/or NO scavengers (Bethke et al., 2011). Indeed, due to the toxicity, reactivity, and gaseous state of NO, direct application is not easy to carry out in the laboratory. Thus, a plethora of compounds known to generate NO are preferentially used instead. All these molecules differ by their characteristics of NO release (kinetic, amount, light-dependency) and can thus lead to contrasted results (Planchet and Kaiser, 2006b). Used in aqueous solutions, NO donors can lead to nitrogen oxides production. In addition, certain of these chemicals are complex molecules with potential side products. For instance, the photolysis of SNP was proved to release more cyanide than NO. Indeed, cyanide may actually be the active compound when applying SNP to seeds (Bethke et al., 2006a). Conversely, the widely used derivatives of PTIO such as c-PTIO are thought to be relatively specific NO scavengers (Akaike et al., 1993): $PTIO + NO \rightarrow PTI + NO_2$. However, the reaction products including PTI may have undesirable side effects in cells (Planchet and Kaiser, 2006a). In a general way, when using NO donors or scavengers, the potential effect of all generated compounds should always be taken into account. The demonstration of opposite effects of NO donors and NO scavengers in a given physiological process is usually considered as a reliable evidence of NO implication.

Methods available for the detection and quantification of nitric oxide in seeds

In animal cells, the absence of nitrate reduction pathways allows the use of assay based on nitrogen oxides, especially nitrite, quantification to evaluate NO production (nitrate and nitrite being considered as by-products of NO production and subsequent oxidation). In plant, such methodology is excluded due to the existence of an active nitrate assimilation pathway responsible for most of nitrite production. Consequently, distinct other methodologies have been applied including fluorescent probes based detection, EPR spectroscopy, electrochemistry, ozone based chemiluminescence, laser photoacoustic, mass spectrometry and the oxyhemoglobin assay. A short discussion on some of these techniques is provided below, for a complete review refer to (Vandelle and Delledonne, 2008; Bethke et al., 2011; Mur et al., 2011).

Several distinct fluorescent probes can be used to investigate NO biosynthesis or release by a given tissue. The diaminofluoresceins (DAF; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein) or the diaminorhodamine 4M (DAR-4M) and their cell permeable forms DAF diacetate (DAF-2DA, DAF-FM DA) and DAR-4M acetoxymethyl ester (DAR-4M AM) are the most commonly used (Kojima et al., 1998, 2001). These probes are sensitive (up to the nM range) but suffer from a serious lack of specificity. Indeed, they do not directly react with NO but with its main oxidation product N_2O_3. Thus, the fluorescence intensity could also depend on the rate of NO oxidation. As the non-enzymatic oxidation of NO requires oxygen, these fluorescent

probes cannot be used under anoxia. Finally, numerous distinct compounds were reported to affect DAF-T fluorescence *in vivo* including ascorbate and dehydroascorbate (Vandelle and Delledonne, 2008). Nonetheless, N_2O_3 detection with DAF-FM was successfully applied on *Arabidopsis* seeds but required to remove the seed testa (Liu et al., 2009).

Electron paramagnetic resonance spectroscopy is a more specific method that can be applied to the direct detection of radical species including NO both *in vitro* and *in vivo*. However, in order to increase its sensitivity, EPR spectroscopy is often associated with the use of spin-traps, molecules that can react with NO and enhance its EPR signal. This technique has been successfully applied to the detection and quantification of NO in embryonic axes homogenates from soybean and sorghum (Caro and Puntarulo, 1999; Simontacchi et al., 2004). However, NO detection from intact seed tissues, eventually supplemented with a spin trap remains a technical challenge as it would require a sufficiently high production to reach the sensitivity threshold.

Another widely used approach is based on the chemiluminescent reaction between gaseous NO and ozone. This technique can allow the direct quantification of NO release from a tissue placed in a sealed compartment under a gaseous flux driving the gas released in the environment to an analyser. NO-specific electrodes are also available but are also difficult to apply to the study of the tiny *Arabidopsis* seeds. They could only be useful to assess the amount of NO released by the seeds in their environment.

Overall, despites all the existing techniques, an accurate detection and quantification of NO generation in plant tissue remain difficult. In addition, most techniques require preparation steps or experimental conditions that can lead to undesirable signal. Thus, as for the pharmacological experiments, a cross validation with at least two distinct quantification methods is highly recommended (Gupta and Igamberdiev, 2013). In case of *Arabidopsis* seeds, the size and characteristics of the mature seeds represents significant technical constraints to an accurate and specific detection/quantification of NO levels by the methodologies currently available.

Genetic resources for the study of nitric oxide production and signaling

The genetic resources available to investigate NO signaling remain restricted due to our limited actual knowledge of NO biosynthesis pathways in plants. Thus, most of the studies rely either on a pharmacological approach (as discussed previously) or on mutants affected in NO availability although their NO levels are not always explained. Some mutants somehow related to NO homeostasis in plants (e.g., *nia1nia2, gsnor, atnoa1)* have been associated to seed phenotypes. However, the interpretation of these phenotypes is often difficult and requires a lot of caution.

Nitrate reductase, being the only identified enzyme proven to be directly involved in NO biosynthesis, NR-deficient mutants has been extensively used, especially the G′4–3 mutant in *Arabidopsis* (Wilkinson and Crawford, 1993). However, NR-deficiency causes important perturbation of nitrogen metabolism and a significant nitrate accumulation resulting in a pleiotropic phenotype (Alboresi et al., 2005). Consequently, it is difficult to establish a direct link between nitrate-related phenotypes and reduced NO production by NR-NiR activity. Moreover, the high nitrate levels could lead to an enhanced NO-independent nitrate-mediated signaling (Alboresi et al., 2005). Contradictory results have been published regarding G′4–3 seeds physiology (Alboresi et al., 2005; Lozano-Juste and Leon, 2010).

Several other mutants known as affected in NO levels have also been used to investigate NO signaling in *Arabidopsis*. Mutants associated to reduced NO levels include NO-Associated 1 (*atnoa1*, At3g47450; Guo et al., 2003) and prohibitin 3 (*phb3*; At5g40770; Wang et al., 2010) while one mutant with enhanced endogenous NO levels was identified as the phosphoenolpyruvate/phosphate translocator chlorophyll a/b binding protein underexpressed 1/NO overproducer 1 (*cue1/nox1*; At5g33320; He et al., 2004). The exact relation between the function of the corresponding proteins and the NO levels in these mutants has not been clearly elucidated yet. Most of these mutants have strong phenotypes but, *phb3* and *cue1/nox1* have not been investigated for seed phenotypes. However, the *atnoa1* mutant has been more studied as it was first proposed as encoding a NO synthase (NOS)-like protein based on sequence similarity with an hypothetical snail NOS and subsequent characterization of a corresponding mutant displaying reduced NOS activity in leaves and lower NO levels in roots (Guo et al., 2003). However, further experiments excluded a direct role for this protein in NO synthesis. Instead, it was later identified as a GTPase. The *atnoa1* mutant seeds were associated with a slightly increased dormancy and a hypersensitivity to salt and osmotic stresses (Zhao et al., 2007; Lozano-Juste and Leon, 2010).

Alternative strategies have been developed to get around the known limitations and pursue the investigations on NO signaling in plants. Promising examples include the use of transgenic lines with altered hemoglobin expression (Perazzolli et al., 2004) and the over-expression of rat neuronal NOS in *Arabidopsis* (Shi et al., 2012). Both strategies already led to significant results even thought all putative side consequences, apart from NO levels alteration, must be considered with extreme caution.

MOLECULAR TARGETS OF NITRIC OXIDE IN SEEDS

Aside from the long lasting question concerning the relevant NO sources in seeds, the re-constitution of NO signaling pathways require the identification of the NO biological targets. Yet, direct molecular targets of NO remain poorly documented in plants. NO could regulate physiological processes by affecting gene transcription. Indeed, several NO-regulated genes, involved in different functional and biological processes, have previously been described (Huang et al., 2002; Polverari et al., 2003; Parani et al., 2004; Grun et al., 2006; Palmieri et al., 2008; Besson-Bard et al., 2009). Furthermore, NO can bind to transition metals of metalloproteins (metal nitrosylation) or cause protein PTMs such as cysteine *S*-nitrosylation or tyrosine nitration (**Figure 2**; Moreau et al., 2010; Arc et al., 2011).

PROTEIN *S*-NITROSYLATION IN SEEDS

Nitric oxide-mediated *S*-nitrosylation of cysteine thiol groups within polypeptide chains is a likely mechanism by which NO may function in signaling processes (Stamler et al., 1992a; Jaffrey et al., 2001). *S*-nitrosylation consists in the covalent attachment of

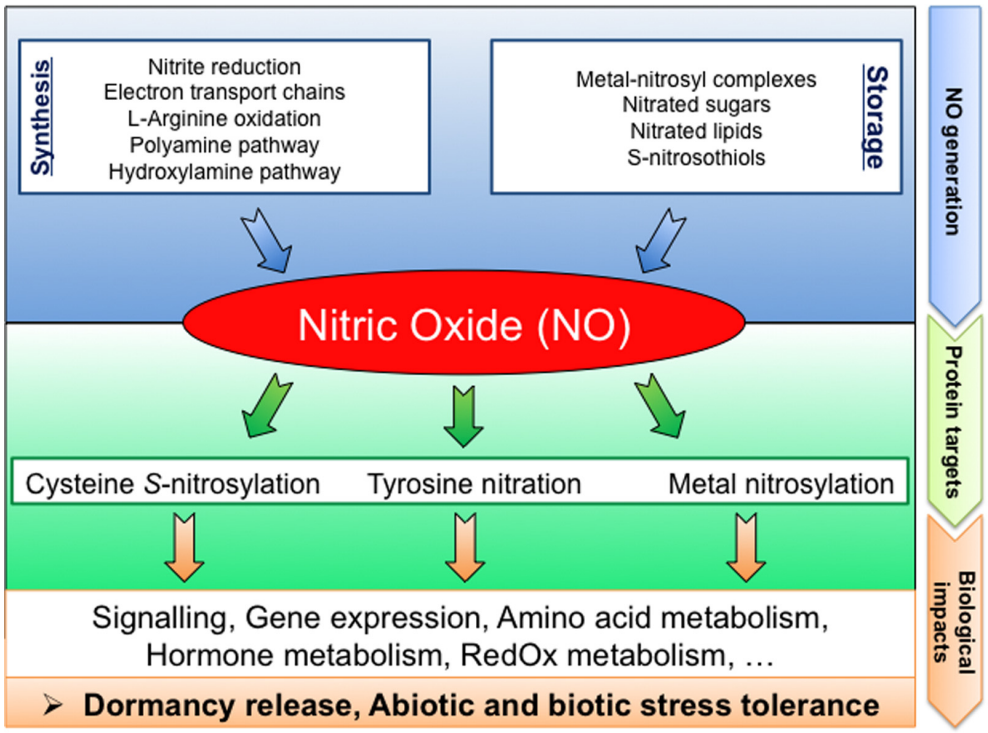

FIGURE 2 | Schematic diagram summarizing NO signaling in seeds: from generation to targets. NO can be produced by various biosynthesis pathways or released from NO-storage compounds. Proteins are preferentially targeted by NO resulting in PTMs (cysteine *S*-nitrosylation, tyrosine nitration, and metal nitrosylation). These NO mediated PTMs modulate the protein functions, leading to strong impacts on cell metabolism thereby affecting seed physiology.

a NO moiety to a reactive cysteine thiol resulting in the formation of a *S*-nitrosothiol group (*S*-NO). In animal systems, regulation of specific proteins by *S*-nitrosylation is an intensively investigated PTM. This PTM, which is thought to be particularly labile, is associated with a precise spatio-temporal regulation and can potentially result in the activation or inactivation of targeted proteins (Hess et al., 2005). It occurs mainly through non-enzymatic reactions being dependent on the physiochemical environment of the protein cysteinyl residues and the proximity of susceptible proteins to NO production sites in cells (Lindermayr and Durner, 2009). On the contrary, protein de-nitrosylation seems to be catalyzed by several enzymes, such as thioredoxins (Trxs) or Cu/Zn superoxide dismutases, as well as by reducing metals and intracellular reducing agents (Lindermayr and Durner, 2009). In fact, because of its selectivity toward protein targets, *S*-nitrosylation may represent a general pathway for modulating protein structure/function, analogs to protein phosphorylation (Spickett et al., 2006). Up to now, only few intracellular *S*-nitrosylated proteins have been identified in plants (Lindermayr et al., 2005; Tanou et al., 2009; Astier et al., 2011; Lounifi et al., 2012). A recent and promising example is the NO-mediated modulation of auxin signaling through the *S*-nitrosylation of the TIR1 auxin receptor. This PTM of TIR1 promotes its interaction with Aux/IAA repressors thereby facilitating their degradation (Terrile et al., 2012). Moreover, NO and ethylene act antagonistically in fruit ripening through inhibition of enzymes involved in ethylene production by *S*-nitrosylation (Manjunatha et al., 2012). In contrast, NO and ethylene act synergistically in seed dormancy release but the underlying molecular mechanisms are still unknown (Gniazdowska et al., 2010b; Arc et al., 2013). Due to the limited permeability of most of their outer layers, seeds can experience hypoxia (Borisjuk and Rolletschek, 2009). Consequently, a fine regulation of oxygen consumption is necessary. This seems to be achieved through NO-mediated inhibition of seed mitochondrial activity (Borisjuk et al., 2007). Consequently, NO-related protein modifications are likely to be increased in seed mitochondria and therefore to play an important role in regulating the activity of these organelles. Many *S*-nitrosylated proteins identified in plants are implicated in metabolic processes (Lindermayr et al., 2005; Abat et al., 2008; Romero-Puertas et al., 2008; Abat and Deswal, 2009; Tanou et al., 2009; Palmieri et al., 2010) suggesting that NO could participate in the regulation of the energy status of the seeds. In agreement, a β-subunit of the mitochondrial ATP synthase complex was found to be *S*-nitrosylated in dry *Arabidopsis* seeds (Arc et al., 2011). Since a homologous protein was shown to be inactivated by *S*-nitrosylation in alcoholic fatty liver of rats (Moon et al., 2006) and more recently in pea leaves mitochondria (Camejo et al., 2013), the seed mitochondrial ATP synthase activity might be inhibited by this NO-mediated PTM. Further experiments are required to assess this hypothesis.

In wheat seeds, a parallel increase in NO and protein *S*-nitrosylation was reported during *sensu stricto* germination

(Sen, 2010). Noteworthy, seed treatments with NO promoted desiccation tolerance, in the recalcitrant species *Antiaris toxicaria*, by limiting protein carbonylation and enhancing protein *S*-nitrosylation (Bai et al., 2011).

PROTEIN NITRATION IN SEEDS

Tyrosine nitration consists in the addition of a nitro group ($-NO_2$) resulting in an alteration of diverse protein functions. The very fast reaction between NO and O_2^- gives rise to peroxynitrite ($ONOO^-$) which is considered as a potent oxidizing and nitrating agent (Ducrocq et al., 1999; Abello et al., 2009). Tyrosine nitration is consequently predominantly observed in states prone to the concomitant release of NO and ROS. Until recently, tyrosine nitration was considered as being irreversible suggesting that the presence of nitrotyrosine in proteins represents a footprint of nitrosative stress. However, increasing evidence suggests the existence of a de-nitration mechanism *in vivo* (Abello et al., 2009). Protein nitration can result in an alteration of diverse protein functions (Alvarez et al., 2011; Melo et al., 2011; Jacques et al., 2012) and could enhance protein sensibility to proteolytic degradation via the proteasome (Abello et al., 2009). Thus, protein nitration would be more than a biological marker of nitrosative stress and could participate in protein turnover or signal transduction in plants (Corpas et al., 2008, 2009; Ischiropoulos, 2009). A single study has been carried out on seeds, more precisely on sorghum embryonic axes (Jasid et al., 2008). This work revealed the appearance of several nitrated proteins upon seed imbibition. A recent study based on immunoprecipitation with an anti-3-nitrotyrosine antibody and subsequent analysis by shotgun liquid chromatography–mass spectrometry (LC-MS/MS) led to the identification of 127 proteins putatively targeted by this PTM in protein extracts from *Arabidopsis* seedlings (Lozano-Juste et al., 2011). Among this important list, a few candidates were further confirmed by additional experiments. Among these numerous putative targets of tyrosine nitration were a few proteins with known implications in seed physiology. For instance, the molybdenum cofactor (MoCo) sulfurase ABA3 (At1g16540) was among these candidates. ABA3 is involved in the last step of ABA synthesis (Mendel, 2007). Thus, the inactivation of ABA synthesis by this PTM might contribute to the control of dormancy release and germination vigor. Overall, nitration may be more than a biological marker of nitrosative stress and could participate in protein turnover or signal transduction in plants (Corpas et al., 2009; Ischiropoulos, 2009). In seeds, the concomitant generation of NO and ROS upon imbibition could lead to enhanced peroxynitrite formation thereby improving tyrosine nitration. Therefore, protein tyrosine nitrations appear likely to occur in this context and in lights of the discussed examples could be of paramount importance.

CONCLUSIONS AND PROSPECTS

Most of the analysis published up to date pinpoint ABA content as a major determinant of dormancy release or maintenance. It appears that the decision to pursue the transition toward germination or maintain a dormant state can be taken during seed imbibition depending on environmental parameters. Thus, the control of ABA levels and sensitivity during early imbibition appears of paramount importance. During this phase, both NO and ROS accumulation has been reported. The intensity of the generation of these radicals could depend on both endogenous and environment cues. In turn, the interplay between ROS and RNS would determine both the extent of ABA catabolism (via the regulation of *CYP707A2* expression for instance) and the sensitivity to this hormone. As a result, theses reactive species could determine the kinetics of ABA degradation and the threshold below which ABA content should fall for germination to occur. As the *de novo* protein synthesis is low during the first hours upon imbibition these effects could be mainly modulated *via* non-enzymatic protein PTMs such as carbonylation, nitration and/or *S*-nitrosylation. Still, both ROS and RNS accumulation can also lead to detrimental damages. Thus, we believe that the concept of "oxidative window" for seed germination should be extended to include NO and associated RNS.

However, despite a general consensus regarding NO importance in seed physiology, the pathways involved in its biosynthesis remain uncertain. This observation presumably reflects the complexity of the regulation of NO biosynthesis in plants. Indeed, multiple different endogenous sources all potentially depending on environmental and/or molecular parameters may contribute to NO accumulation in seeds. Moreover, the relevant reactions in seeds may be significantly different from those described at other physiological stages including the non-enzymatic reactions that may occur in the apoplast next to the aleurone layer (Bethke et al., 2004a). To discriminate between the relative contribution of the distinct known NO sources, accurate determination of NO content in seeds and especially during imbibition appears absolutely required. However, the relatively low amount of NO released under physiological conditions and the drawbacks of the techniques currently available makes NO measurement a very challenging issue. In any case, an unambiguous confirmation of NO accumulation in the seed endosperm and/or embryo appears as a priority to consolidate the available evidences and determine the seed NO content.

In a similar way, we are firmly convinced that NO-related PTMs, namely tyrosine nitration and cysteine *S*-nitrosylation, can explain the effect of NO in seeds though this assumption is not totally confirmed yet. Indeed, the detection and identification methods for both cysteine *S*-nitrosylation and tyrosine nitration proved difficult to apply on seeds most presumably due to the low abundance of modified proteins and/or the limited stability of the modifications. Nonetheless, these two PTMs represent very seducing models to explain the roles ascribed to NO in seeds. The characterization of NO-targeted proteins in various seed physiology context will undoubtedly reveal new area of research to explore for understanding the control of germination.

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Possible role of glutamine synthetase in the NO signaling response in root nodules by contributing to the antioxidant defenses

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Nitric oxide (NO) is emerging as an important regulatory player in the *Rhizobium*-legume symbiosis. The occurrence of NO during several steps of the symbiotic interaction suggests an important, but yet unknown, signaling role of this molecule for root nodule formation and functioning. The identification of the molecular targets of NO is key for the assembly of the signal transduction cascade that will ultimately help to unravel NO function. We have recently shown that the key nitrogen assimilatory enzyme glutamine synthetase (GS) is a molecular target of NO in root nodules of *Medicago truncatula*, being post-translationally regulated by tyrosine nitration in relation to nitrogen fixation. In functional nodules of *M. truncatula* NO formation has been located in the bacteroid containing cells of the fixation zone, where the ammonium generated by bacterial nitrogenase is released to the plant cytosol and assimilated into the organic pools by plant GS. We propose that the NO-mediated GS post-translational inactivation is connected to nitrogenase inhibition induced by NO and is related to metabolite channeling to boost the nodule antioxidant defenses. Glutamate, a substrate for GS activity is also the precursor for the synthesis of glutathione (GSH), which is highly abundant in root nodules of several plant species and known to play a major role in the antioxidant defense participating in the ascorbate/GSH cycle. Existing evidence suggests that upon NO-mediated GS inhibition, glutamate could be channeled for the synthesis of GSH. According to this hypothesis, GS would be involved in the NO-signaling responses in root nodules and the NO-signaling events would meet the nodule metabolic pathways to provide an adaptive response to the inhibition of symbiotic nitrogen fixation by reactive nitrogen species.

Keywords: root nodules, nitrogen fixation, glutamine synthetase, tyrosine nitration, nitric oxide, *Medicago truncatula*

INTRODUCTION

Leguminous plants associated with symbiotic bacteria of the family *Rhizobiaceae* are able to grow under nitrogen-limiting conditions. Key to this achievement is the bacterial ability to reduce atmospheric nitrogen in a functional symbiotic interaction, in which ammonia is provided to the plant and assimilated into organic composition by the plant enzyme glutamine synthetase (GS; EC 6.3.1.2). The establishment of this symbiosis requires a constant fine-tuned signal exchange between plant and bacteria culminating with the formation of a novel organ, the root nodule, which provide an environment suitable for bacterial nitrogen fixation (Oldroyd et al., 2011). Symbiotic nitrogen fixation is of particular agricultural and ecological importance, as it constitutes one of the largest contributions to biologically available nitrogen in the biosphere. Therefore, the identification of the regulatory signaling network underlying the symbiotic interaction is of utmost importance and has been the subject of intense research (for recent reviews, see Oldroyd et al., 2011; Udvardi and Poole, 2013). In recent years, nitric oxide (NO), widely recognized as an endogenous signaling molecule, emerged as an important player in the legume–rhizobium interaction, but its mechanisms of action are still far from being understood (Besson-Bard et al., 2008; Neill et al., 2008; Meilhoc et al., 2011; Puppo et al., 2013). To unravel the signal transduction cascade and ultimately NO function, it is necessary to identify its molecular targets. We have recently shown that GS, a key enzyme for nodule functioning, is a molecular target of NO, being post-translationally regulated by tyrosine nitration in relation to active nitrogen fixation (Melo et al., 2011). In functional nodules of *Medicago truncatula* NO production has been located in the bacteroid containing cells of the nodule fixation zone (Baudouin et al., 2006), where glutamine synthetase is highly abundant. The location of the enzyme at the sites of NO production together with its position at the center of the complex matrix of nitrogen metabolism conveys an important role of the enzyme at the crossroads of signaling events. We propose that the regulation of glutamine synthetase by NO is related to metabolite channeling to boost the nodule antioxidant defenses, linking NO signaling with nitrogen metabolism. This article discusses this hypothesis in view of the existing evidence supporting a role of glutamine synthetase in the NO signaling cascade in root nodules.

EVIDENCE FOR A SIGNALING ROLE OF NO IN THE SYMBIOTIC INTERACTION

The formation of NO and its involvement in the legume-rhizobia symbiosis has been the subject of much research in the last few years. It is now well established that the molecule is produced in root nodules and is important both for nodule development and functioning (Meilhoc et al., 2011; Wang and Ruby, 2011; Puppo et al., 2013). Nodule formation is highly complex and involves a progression of temporally and spatially regulated events, which require extensive recognition and signaling by both partners. The first signal is plant-released flavonoids and related compounds, which elicit synthesis of lipochito-oligosaccharides (Nod factors) by rhizobia. Nod factors induce cell division in the inner root cortex and the formation of a nodule primordium. In parallel, bacteria enter the root hairs via infection threads, are released to the plant cells by endocytosis and remain surrounded by a plant-derived symbiosome membrane (Oldroyd et al., 2011). As the nodule primordia continue to grow, new plant cells are continuously being infected and fully developed legume nodules contain a large central tissue harboring thousands of nitrogen fixing bacteria. The fixed nitrogen is exported as ammonium to the plant cytosol where it is assimilated into organic compounds by plant GS. In exchange for reduced nitrogen from the bacteria, the plant provides rhizobia with reduced carbon and all the essential nutrients required for bacterial metabolism. As nitrogenase is strongly inhibited by oxygen, nitrogen fixation is made possible by the microaerophilic conditions prevailing in the nodule, where the oxygen concentration is controlled by a variable-permeability barrier in the nodule parenchyma and by leghemoglobin, an oxygen-binding plant protein regulating and delivering oxygen to the infected cells (Udvardi and Poole, 2013). As such, the process of nodulation involves infection, development and metabolic processes and the signals exchanged between the two partners will encompass very different physiological contexts. NO is known to be involved in physiological processes ranging from biotic and abiotic stress responses, to normal plant growth and development (Besson-Bard et al., 2008). A number of reports document that NO is involved in the signaling network in root nodules, both at the early steps of plant–bacteria interaction and at later stages in mature nitrogen-fixing nodules, suggesting distinct roles of the molecule at different steps of the symbiosis. This subject has been reviewed comprehensively elsewhere (Meilhoc et al., 2011; Wang and Ruby, 2011; Puppo et al., 2013). Here we recapitulate very briefly the recent disclosures obtained using the model legume *Medicago truncatula* and its symbiotic partner *Sinorhizobium meliloti*.

During early steps of the *M. truncatula–S. meliloti* interaction, NO has been detected both at the infection sites and in the nodule primordia, suggesting an involvement of NO in both bacterial infection and nodule organogenesis. Evidence for an important role of the molecule in nodule formation was given by the finding that NO depletion resulted in a significant delay in nodule appearance and provoked the down regulation of genes involved in nodule development (del Giudice et al., 2011). In fully developed root nodules NO has been located exclusively in the infected cells and appears to be confined to the nodule fixation zone, pointing to an involvement of the molecule in root nodule metabolism (Baudouin et al., 2006; Horchani et al., 2011). A metabolic function for NO in providing a significant energy input in mature nitrogen-fixing nodules through the nitrate-NO respiration process has been recently highlighted (Horchani et al., 2011). NO has also been shown to modulate the expression of a wide number of genes both from *S. meliloti* (Meilhoc et al., 2010) and *M. truncatula* (Ferrarini et al., 2008). Many of the NO-responsive *M. truncatula* genes are involved in nodule development and functioning, with a significant number of the NO-responsive genes being involved in primary metabolism, further supporting a signaling role of NO in the nodule metabolic pathways (Ferrarini et al., 2008). More recently, NO production has also been associated with nodule senescence. Using both genetic and pharmacological approaches, it was shown that NO accumulation in aging nodules of *M. truncatula* has deleterious effects on the symbiosis by inhibiting nitrogen fixation and activating nodule senescence, whereas a decrease in NO levels leads to a delay in nodule senescence (Cam et al., 2012).

The origin of NO in plants is still not clearly understood, and in root nodules the picture is even more complex because the source of NO is probably variable at different stages of the symbiotic interaction and can arise from both symbiotic partners (Meilhoc et al., 2011). Several routes capable of yielding NO in root nodules have been described: NO synthase (NOS)-like activity converting arginine to citrulline and NO (Cueto et al., 1996; Baudouin et al., 2006; Leach et al., 2010), and nitrate reductase and the electron transfer chains from both plants and bacteria (Mesa et al., 2004; Meakin et al., 2007; Gupta et al., 2011a; Horchani et al., 2011).

Nitric oxide can signal fundamental physiological processes by changing both gene expression and protein function and a major step towards understanding the mechanisms regulated by NO during the symbiosis relies on the identification of its molecular targets. This task is made difficult, because the physiological contexts underlying discrete symbiotic stages are highly variable, ranging from infection, to development and senescence and thus the molecular targets of NO are expected to vary at different stages of the symbiotic interaction. While considerable effort is being put forward to identify the molecular targets of NO using large scale approaches, either by proteomics (Cecconi et al., 2009; Chaki et al., 2009; Lozano-Juste et al., 2011) or transcriptomics (Ferrarini et al., 2008; De Michele et al., 2009; Boscari et al., 2013), GS was identified as a molecular target of NO by a simple biochemical approach (Melo et al., 2011).

EVIDENCE FOR A CRUCIAL ROLE OF THE NODULE ANTIOXIDANT RESPONSES IN NITROGEN FIXATION

Whilst it is now evident that NO is required for nodule functioning, paradoxically it is also clear that it is a potent inhibitor of nitrogenase activity (Trinchant and Rigaud, 1982; Kato et al., 2010). The involvement of NO in nitrogenase inactivation has been demonstrated in soybean and *Lotus* after nitrate supply (Kanayama et al., 1990; Meakin et al., 2007; Kato et al., 2010). In *Lotus japonicus*, the artificial application of the NO donor sodium nitroprusside (SNP) decreased nitrogen fixation, whereas the application of a NO scavenger (cPTIO) had the opposite effect (Shimoda et al., 2009; Kato et al., 2010). Thus, the NO concentration inside the nodule needs

to be maintained at levels compatible with nitrogenase activity, but still be sufficient to achieve its signaling function. This implies a balance between NO production and detoxification. The plant antioxidant responses are therefore of crucial importance to maintain nodule functioning (Pauly et al., 2006; Becana et al., 2010; Sanchez et al., 2011). Most of the antioxidants in legume nodules are also present in other plant organs or tissues, but the concentrations in nodules are generally higher, denoting a connection between N_2 fixation and the antioxidant response (Puppo et al., 2013). The data published to date indicate that hemoglobins (Hbs) and the GSH/ascorbate pathway constitute the chief antioxidant mechanisms in root nodules (Becana et al., 2010) and will be considered separately.

HEMOGLOBINS

The levels of NO inside the nodule appear to be controlled by Hbs, which are able to scavenge NO, and in this way may protect nitrogenase from inactivation. In legumes, three types of Hb have been described: symbiotic Hb (Lb), non-symbiotic Hb (nsHb) and truncated Hb (trHb; Bustos-Sanmamed et al., 2011). The nsHbs are subdivided into nsHb-1s (class 1 nsHbs), which have a very high affinity for O_2, and nsHb-2s (class 2 nsHbs), which have lower affinity for O_2 and are similar to the sHbs (Gupta et al., 2011b). The first evidence of NO binding to Hb was given by the detection of nitroso-leghemoglobin complexes (LbNO) in nodules of soybean and Lotus (Kanayama et al., 1990; Mathieu et al., 1998; Meakin et al., 2007; Sanchez et al., 2010). Later, this NO-scavenging function has also been attributed to non-symbiotic class 1 Hbs (nsHb1) in *Lotus japonicus* (Shimoda et al., 2009) and more recently the three types of Hb were found to be expressed in nodules of *Lotus japonicus*, suggesting complementary roles of the different types of Hb for root nodule formation and/or functioning (Bustos-Sanmamed et al., 2011). Because class 1 nsHbs have an extreme affinity for O_2, it is unlikely that they function as O_2 transporters, stores, or sensors, therefore they have been supposed to play the role of NO scavenger in NO detoxifying pathways (Gupta et al., 2011b; Igamberdiev et al., 2011). These proteins are induced upon symbiotic infection, accumulate in nitrogen fixing nodules and their over-expression enhances symbiotic N_2 fixation, further supporting a role in NO quenching in root nodules (Shimoda et al., 2009). An NO scavenging role has also been attributed to the flavohemoprotein Hmp of the bacterial partner (Meilhoc et al., 2010). Indeed, using *S. meliloti hmp* mutant strains and Hmp over-expressing strains, it was recently shown that this protein can modulate the levels of NO inside the nodules (Cam et al., 2012). A direct relationship between NO scavenging by Hbs and nitrogen fixation is reinforced by the fact that the over-expression of either plant ns-Hb1 in the plant partner (Nagata et al., 2008; Shimoda et al., 2009) or bacterial Hbs in the rhizobial partner (Ramirez et al., 1999; Cam et al., 2012) lead to enhanced symbiotic N_2 fixation, whereas this process is impaired in rhizobial *hmp*$^-$ mutants in *M. truncatula* (Meilhoc et al., 2010; Cam et al., 2012). All together, the available data suggest that both the plant and the bacterial Hbs are involved in the signaling responses to NO and are important for N metabolism in root nodules.

GSH/ASCORBATE CYCLE

The GSH/ascorbate pathway provides one of the main antioxidant mechanisms in plants and several lines of evidence indicate that this pathway is a major contributor to the antioxidant defenses in nodules (reviewed in Matamoros et al., 2003; Pauly et al., 2006; Becana et al., 2010; Puppo et al., 2013). In legume root nodules there is a close positive correlation between nitrogenase activity, ascorbate and glutathione (GSH)/homoglutathione content (Dalton et al., 1993; Matamoros et al., 2003; El Msehli et al., 2011). The thiol tripeptides GSH and hGSH are known to be at high concentrations in nodules and to play key roles in both nodule formation and functioning (Frendo et al., 2005; Pauly et al., 2006; El Msehli et al., 2011). The substrates for GSH and hGSH synthesis are glutamate and cysteine and the pathway involves two ATP-dependent steps. In the first reaction, γ-glutamyl-cysteine synthetase (γ ECS; EC 6.3.2.2) catalyses the formation of γ-glutamylcysteine, and in the second reaction, glycine or β-alanine is added to the C-terminal site of γ-glutamylcysteine by GSH synthetase (GSHS; EC 6.3.2.3) or hGSH synthetase (hGSHS), respectively (Frendo et al., 1999, 2001). Recently, it was shown that GSHS and hGSHS follow a tissue-specific pattern of expression in the nodules of *M. truncatula*, pointing to a tissue-specific differential regulation of GSH and hGSH synthesis in *M. truncatula* (El Msehli et al., 2011). The importance of (h)GSH for nitrogen fixation was recently evidenced by studies in transgenic nodules with decreased or increased (h)GSH content in the nitrogen-fixing zone. These studies showed that the concentration of (h)GSH regulates nitrogen fixation efficiency and that a deficiency in (h)GSH impairs nodule growth (El Msehli et al., 2011).

Glutathione can readily react with NO to form S-nitrosoglutathione (GSNO) and may play an important role in regulating NO bioactivity. While the half-life of NO in biological systems is only a few seconds, GSNO is relatively stable and thought to function as a NO reservoir, since it can release NO or function as a transnitrosylating agent. The key enzyme regulating GSNO pools is S-nitrosoglutathione reductase (GSNOR), reducing GSNO to ultimately produce glutathione disulfide (GSSG), which can be reduced by glutathione reductase (GR) to re-enter the GSH pool and ammonia, which can be re-assimilated by GS (Liu et al., 2001; Lamotte et al., 2005).

Interestingly, it was reported that GSH is produced in response to elevated NO in roots of *M. truncatula* (Innocenti et al., 2007). As GS activity is inhibited by NO and one of its substrates, glutamate is also a substrate for (h)GSH synthesis, we proposed that upon NO-induced inhibition of GS, glutamate could be channeled to the synthesis of (h)GSH, contributing in this way, to the nodule antioxidant defenses and to the protection of nitrogenase from inactivation by NO. This aspect will be further discussed in the last section of this article.

EVIDENCE FOR THE REGULATION OF GLUTAMINE SYNTHETASE ACTIVITY BY NO

Glutamine synthetase is abundantly present in root nodules where it plays a pivotal role in the assimilation of the ammonium released by nitrogen fixation. The enzyme catalyses the ATP-dependent condensation of ammonium with glutamate to yield glutamine, which can be directly exported from the nodules or used to

synthetize asparagine, the main nitrogen export compound in indeterminate nodules (Vance, 2008). In the model legume *M. truncatula* GS is encoded by four expressed genes, two (*MtGS1a* and *MtGS1b*) encoding cytosolic isoenzymes, and two (*MtGS2a* and *MtGS2b*) encoding plastid located isoenzymes (Stanford et al., 1993; Carvalho et al., 2000a,b; Melo et al., 2003; Seabra et al., 2010), the latter of which is exclusively expressed in the seeds and is unique to *M. truncatula* and closely related species (Seabra et al., 2010). The other three GS genes are expressed in root nodules, but *MtGS1a* is highly up regulated, accounting for the production of over 90% of the total nodule GS activity, and encodes the isoenzyme responsible for the assimilation of the ammonia released by nitrogen fixation (Carvalho et al., 2000a). We have previously shown that MtGS1a is abundantly present in the infected cells of the nodule fixation zone (Carvalho et al., 2000a), coinciding with the major site of NO formation in this model species (Baudouin et al., 2006; Horchani et al., 2011). The enzyme is thus *in vivo* accessible to the oxidative effects induced by this reactive compound and it was shown to be a molecular target of NO in root nodules (Melo et al., 2011). *In vitro* studies using purified recombinant enzymes, demonstrated that the *M. truncatula* nodule enzyme MtGS1a is subjected to tyrosine nitration and that this modification provokes a total loss of enzyme activity (Melo et al., 2011). It is noteworthy that the plastid located GS isoenzyme, MtGS2a, which is also expressed in root nodules but at considerably lower levels, is also affected by NO, but by a different mechanism, cysteine nitrosylation. The finding that two isoenzymes that share a high degree of sequence homology and a remarkably conserved active site fold are differentially modified by NO, strengthens the idea that the NO signaling effects are specific under different physiological contexts. In addition to a differential sensitivity of individual GS isoenzymes to NO, the differential localization of the isoenzymes in specific organelles and/or plant tissues is likely to be implied in the NO-mediated regulation of GS activity. Future studies should address the regulation of the plastid located GS isoenzyme by S-nitrosylation. The enzyme is also expressed in the infected cells of root nodules and its expression is positively correlated with active nitrogen fixation (Melo et al., 2003). Here, we will focus on the regulation of MtGS1a by NO, because it is the *M. truncatula* GS isoenzyme responsible for the assimilation of the ammonium released by bacterial nitrogenase.

MECHANISTIC OF MtGS1a INACTIVATION BY TYROSINE NITRATION

Protein tyrosine nitration is a post-translational modification (PTM) mediated by reactive nitrogen species (RNS), resulting from the addition of a nitro ($–NO_2$) group to one of two equivalent ortho carbons in the aromatic ring of tyrosine residues (Radi, 2004). The incorporation of a nitro group ($–NO_2$) into protein tyrosines can lead to profound structural and functional changes, the most common being loss of function (Radi, 2013). This PTM has been best studied in animals and it is a relatively new area of research in higher plants. A number of nitrated proteins have been identified in plants by proteomic approaches (Cecconi et al., 2009; Chaki et al., 2009; Lozano-Juste et al., 2011; Begara-Morales et al., 2013), however the functional effects of nitration on specific proteins are Known only for a few plant proteins (Lozano-Juste et al., 2011; Corpas et al., 2013) and the physiological significance of this PTM remains largely unknown. The tyrosine nitration of MtGS1a has become a good case study on how nitration of tyrosines can promote conformational changes leading to a loss of function. Furthermore, the nitration of MtGS1a in root nodules negatively correlates with active nitrogen fixation, strongly suggesting that the nitration of the enzyme is physiologically relevant for root nodule functioning, an aspect that will be further discussed in the next section.

Tyrosine nitration is considered a selective process, and typically only one or two of the tyrosine residues present in a protein become preferentially nitrated, depending on the structural environment (Abello et al., 2009). By site-directed mutagenesis it was shown that at least two of the 19 tyrosine residues of MtGS1a are prone to nitration, as the substitution of either Tyr 167 or Tyr 263 to phenylalanine reduced by half the protein anti-nitrotyrosine immunoreactivity. However, only mutation on Tyr 167 results in a significant reduction in the NO-mediated inhibitory effect, thus indicating that it is the relevant regulatory site (Melo et al., 2011). Since the three-dimensional structure of MtGS1a is available (Seabra et al., 2009), it was possible to enlighten the structural basis by which the nitration of Tyr167 leads to enzyme inactivation. An analysis of the structural environment of Tyr167 revealed that this residue is located in a solvent-accessible loop, close to the enzyme active site and in close proximity to a basic residue (Lys-137). In the *M. truncatula* enzyme, Tyr-167 establishes a hydrogen bond with Lys-137, and the nitration of this residue could prevent the formation of this bond, which appears to be important to maintain a correct conformation of the active site and is expected to interfere with the catalytic activity of the enzyme (**Figure 1**). Thus, the mechanism of MtGS1a inactivation by tyrosine nitration can be elucidated in structural terms. A rare example, since protein tyrosine nitration is a non-enzymatic mechanism based on free radical reactions and its selectivity for target residues in proteins is far from obvious.

PHYSIOLOGICAL SIGNIFICANCE OF GS NITRATION FOR ROOT NODULE FUNCTIONING

Glutamine synthetase in conjunction with NADH-glutamate synthase (NADH-GOGAT, EC 1.4.1.14) operates the GOGAT cycle leading to the synthesis of glutamine and glutamate, which then serve as nitrogen donors for the biosynthesis of essentially all nitrogenous compounds. In temperate legumes, fixed nitrogen is exported from the nodules to the rest of the plant mainly as asparagine, which is synthesized by the concerted action of two additional enzymes, aspartate aminotransferase (AAT, EC 2.6.1.1) and asparagine synthetase (AS, EC 6.3.5.4). Being the first enzyme of the pathway, GS is placed in a key position to play a regulatory role in the nitrogen assimilatory pathways in nodules. The finding that it is a molecular target of NO, is thus particularly interesting. The formation of NO by plants is necessarily closely linked to nitrogen metabolism, since it is produced from inorganic (reduction of nitrate via nitrite; Horchani et al., 2011), or organic nitrogen sources like arginine via NOS-like activity (Cueto et al., 1996; Baudouin et al., 2006) and potentially polyamines, via a yet non-identified polyamine oxidation pathway (Gupta et al., 2011a;

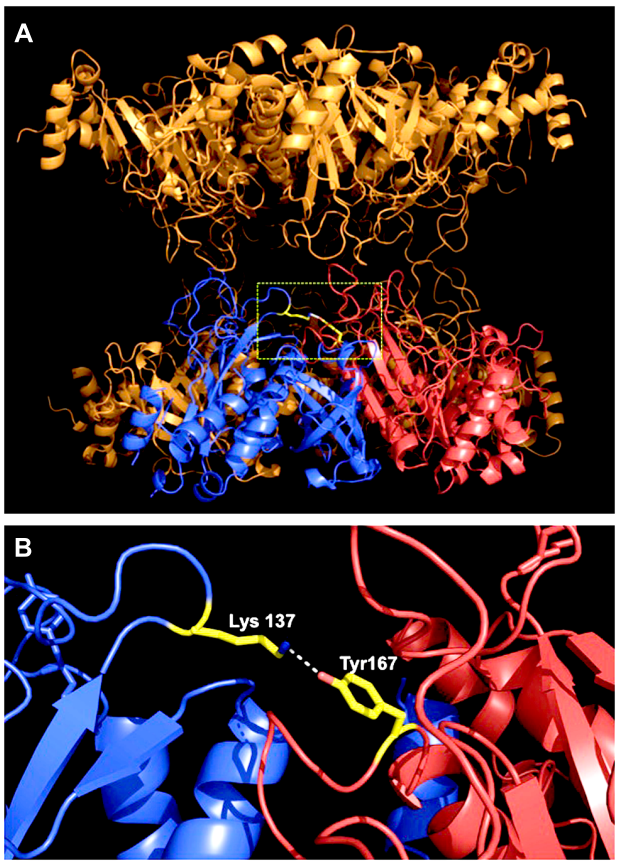

FIGURE 1 | Location of the regulatory nitration site within the three-dimensional structure of MtGS1a. (A) Side-view of the MtGS1a molecule, which is a decamer composed of two stacked (face-to-face) pentameric rings, with 10 active sites formed between the C-terminal domain of one subunit and the N-terminal domain of the other subunit within a pentameric ring (Seabra et al., 2009). The position of Tyr167 is shown in yellow, in a solvent-accessible loop at the interface between two neighboring subunits, which are colored blue and red. **(B)** Arrangement of two neighboring subunits, highlighting the position of tyrosine 167 of the subunit labeled in red, close to the enzyme active site, and establishing an hydrogen bound with Lys-137 of the neighboring subunit, which is presented in blue.

Meilhoc et al., 2011). Research in nodules of *M. truncatula* established that NO accumulation is not a by-product of symbiotic nitrogen fixation (Baudouin et al., 2006), however any of the NO generating pathways that have been described will require adequate nitrogen supply at the sites of NO production. GS is a key enzyme in nitrogen metabolism and in addition to its vital role in primary N assimilation is also crucial in N recycling in plants. Therefore, the regulation of GS by NO establishes a connection between NO signaling and N metabolism.

Nitric oxide is a strong inhibitor of nitrogenase activity, and it seems reasonable that the same signaling molecule also inhibits GS, the enzyme that uses the product of nitrogenase activity as a substrate. Indeed, in root nodules of *M. truncatula* it was shown that GS is inactivated by tyrosine nitration *in planta* and that the GS nitration status is positively correlated with the inhibition of nitrogen fixation (Melo et al., 2011). The GS nitration status was quantified *in planta* in situations where nitrogen fixation is impaired and NO is known to be produced, namely in ineffective nodules, induced either by *nifH*- or *fixJ*-rhizobial strains, as well as in nodules fed with nitrate or treated with the NO donor SNP. A direct relationship could be established between increased GS nitration, reduced nodule GS activity and reduced nitrogen fixation activity, strongly suggesting that GS is post-translationally inactivated by NO-mediated nitration in response to lower nitrogen fixation rates (Melo et al., 2011). NO concentration is expected to raise in root nodules following nitrate application (Kanayama et al., 1990; Kato et al., 2010), however in ineffective nodules NO production appears to be unaffected (Baudouin et al., 2006) and thus it seems that the regulation of GS activity by tyrosine nitration is a specific process associated with nitrogen fixation rather than a general effect resulting from increased NO levels inside the nodule.

Additional evidence for a specific regulation of GS by NO in root nodules is given by recent studies using *S. meliloti* strains carrying a mutation in the gene encoding flavohemoglobin (*hmp*), which is involved in NO degradation and leads to increased NO content inside the nodules(Meilhoc et al., 2010; Cam et al., 2012). Quantification of GS nitration in *hmp*$^-$ mutant nodules revealed a considerable increase in GS nitration in relation to wild type nodules, with a concomitant decrease in GS activity (H. Carvalho, unpublished results). As it has been shown that nodules formed by the *hmp*$^-$ mutant rhizobium suffer a premature senescence induced by NO (Cam et al., 2012), it is tempting to speculate that the NO-induced GS inhibition could be associated with this premature nodule senescence. This idea is supported by the finding that the application of the GS inhibitor phosphinothricin (PPT) to root nodules promotes nodule senescence (Seabra et al., 2012).

The finding that the root enzymes appear to respond differently to NO also supports a specific role of GS in the NO signaling response in root nodules. Following nitrate supply, the GS nitration status was found to be unaffected in roots but increased in root nodules (Melo et al., 2011). The total amount of nitrated proteins, which was quantified by direct ELISA using a specific anti-nitrotyrosine antibody, increases in both the roots and the nodules following nitrate supply, but GS does not appear to be among these proteins in the roots (H. Carvalho, unpublished results). It is noteworthy that in *M. truncatula* roots, GS is mainly composed of a different cytosolic isoenzyme, MtGS1b, which is largely located in the root cortex, whereas MtGS1a is confined to the root vascular tissues (Carvalho et al., 2000b). It is probable that both the formation of NO at the sites of expression of each individual GS isoenzyme and the differential sensitivity of the two isoenzymes to NO account for the differential regulation of GS in roots and root nodules. This is in agreement with the general idea that the effects of NO are not simply a consequence of the amount of NO produced but, are determined by the local environment in which NO is released and the nature of the generated RNS, which in turn will be dependent of the cellular redox state, the bioavailability of NO-generating enzyme substrates, the nature and proximity of molecular targets and of NO-metabolizing proteins.

Taken together, the available information supports a role of NO in mediating GS activity in root nodules as a function of the

nitrogen fixation status, rather than this being a consequence of a general increase in NO concentration inside the nodule.

PROPOSED MODEL FOR THE INVOLVEMENT OF GS IN THE NO SIGNALING PATHWAY IN ROOT NODULES

We hypothesize that the inactivation of GS by tyrosine nitration is an NO-mediated regulatory process important for nodule functioning. In view of the overall available evidence, which was described in the previous sections, we propose a model to explain the involvement of GS in the NO signaling pathway in root nodules (**Figure 2**). According to this model, the inhibition of GS activity by tyrosine nitration would be directly related to the NO-induced nitrogenase inhibition. In view of the fact that elevated levels of NO in root nodules lead to decreased production of ammonium for GS assimilation, the enzyme would be shut down by post-translational inactivation through tyrosine nitration in response to the signal NO, the same signal that shuts down nitrogenase. This way, NO would be placed as a regulatory molecule coordinating N fixation and assimilation in root nodules.

We further propose that the NO-induced GS inhibition is involved in the nodule antioxidant response to NO and related RNS. Glutamate, a substrate for GS activity is also the precursor for the synthesis of GSH, which as described in a previous section, is known to be highly abundant in root nodules of several plant species and to play a major role in the antioxidant defense participating in the GSH/ascorbate cycle (Matamoros et al., 1999b, 2003; Becana et al., 2010). Upon NO-mediated GS inhibition, glutamate could be channeled for the synthesis of GSH contributing to neutralize the deleterious effects of RNS. This idea is supported by the finding that the synthesis of the two enzymes involved in GSH production from glutamate, γ-glutamylcysteine synthetase (γ-ECS) and GSHS is up regulated by NO in *M. truncatula*, correlating with the accumulation of the end product GSH (Innocenti et al., 2007). According to this theory, GS would be involved in the NO signaling pathway, functioning both as a sensor of increased levels of NO inside the nodules and as an activator, by forcing the N metabolic pathways to shift from primary N assimilation to the synthesis of GSH. This, in turn, would boost the nodule antioxidant responses and adjust the levels of NO inside the nodule. Since GSNO, formed by the reaction of NO with GSH, is thought to function as a mobile reservoir of NO bioactivity, GS would play an additional share in the NO signaling cascade by contributing to storage of the signaling molecule in the form of GSNO. NO release from GSNO would then be controlled by the enzyme GSNOR (Leterrier et al., 2011).

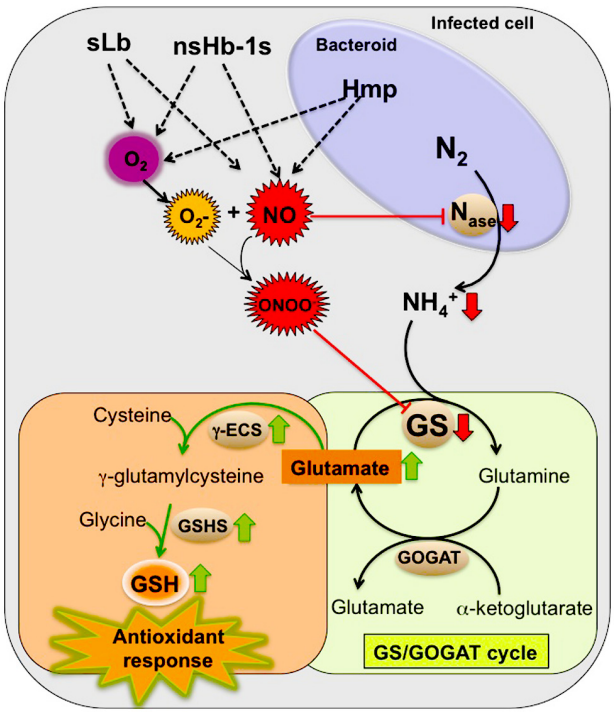

FIGURE 2 | Proposed model for the involvement of GS in the NO signaling events in root nodules by contributing to the nodule antioxidant responses. Red arrows indicate down regulation by NO and the green arrows indicate up regulation by NO. Enzymes: nitrogenase (Nase), glutamine synthetase (GS), glutamate synthase (GOGAT), γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSHS). Hemoglobins: symbiotic leghemoglobin (sLb), class 1 non-symbiotic hemoglobins (nsHb-1s), rhizobial flavohemoprotein (Hmp).

The proposed model also predicts that Hbs are important players in the process by regulating the levels of both O2 and NO, which may compete for binding sites, controlling in this way, the formation of peroxynitrite (ONOO–). Peroxynitrite is probably the main nitrating agent *in vivo* and is formed rapidly in the reaction of the superoxide anion (O_2^-) with NO (Abello et al., 2009; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). As discussed before, it is documented that at least three types of Hbs have the capacity to scavenge NO, contributing in this way to modulate NO bioactivity and protecting nitrogenase from inactivation (Kanayama et al., 1990; Herold and Puppo, 2005; Meakin et al., 2007; Sanchez et al., 2010). We thus anticipate the participation from the plant side, of leghemoglobin and non-symbiotic Hb, pointing to class1 nsHb as the best candidates, and from the bacterial side the flavohemoprotein Hmp.

According to the proposed model, the NO-signaling events would meet the nodule metabolic pathways to provide an adaptive response to the inhibition of symbiotic nitrogen fixation by RNS.

CONCLUSION

Post-translational nitration of key enzymes and the subsequent alteration of their catalytic properties may represent a new level of regulation of primary metabolism. Here we propose that the key nitrogen metabolic enzyme, glutamine synthetase is involved in the NO signaling pathways in root nodules by shifting primary N assimilation to the production of GSH in response to increased NO. For a signaling molecule to be effective, it needs to be produced quickly on demand, induce defined effects within a cell and to be removed rapidly and effectively when it is no longer required. According to the proposed hypothesis, GS would be involved in NO sensing and removal and also in NO storage by controlling GSNO pools. This mechanism would be important, on one hand to coordinate N-fixation and assimilation in the nodules and on the other hand, to boost the antioxidant defenses of the nodule in response to NO. The proposed model conveys an important role for the enzyme at the crossroads of signaling events,

connecting nitrogen metabolism to NO production, storage and detoxification.

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Protein *S*-nitrosylation in plants under abiotic stress: an overview

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Abiotic stress is one of the main problems affecting agricultural losses, and understanding the mechanisms behind plant tolerance and stress response will help us to develop new means of strengthening fruitful agronomy. The mechanisms of plant stress response are complex. Data obtained by experimental procedures are sometimes contradictory, depending on the species, strength, and timing applied. In recent years nitric oxide has been identified as a key signaling molecule involved in most plant responses to abiotic stress, either indirectly through gene activation or interaction with reactive oxygen species and hormones; or else directly, as a result of modifying enzyme activities mainly by nitration and *S*-nitrosylation. While the functional relevance of the *S*-nitrosylation of certain proteins has been assessed in response to biotic stress, it has yet to be characterized under abiotic stress. Here, we review initial works about *S*-nitrosylation in response to abiotic stress to conclude with a brief overview, and discuss further perspectives to obtain a clear outlook of the relevance of *S*-nitrosylation in plant response to abiotic stress.

Keywords: abiotic stress, nitric oxide, plant, post-translational modifications, *S*-nitrosylation

INTRODUCTION

During their life, plants are continuously exposed to extremes in environmental conditions, in particular abiotic stress sources such as drought, high or low temperatures, high salinity, heavy metal exposure, or herbicides affecting plant development and production (Mittler, 2006; Sreenivasulu et al., 2007). Over 90% of the world's arable lands are reportedly exposed to major environmental stresses (Pinho dos Reis et al., 2012). Plants use complex recognition and response mechanisms to protect themselves from environment-related changes (Yamaguchi-Shinozaki and Shinozaki, 2006). The effects of abiotic stress may be general or non-specific, such as growth inhibition, electrolyte leakage, and excess of reactive oxygen species (ROS), all of which could lead to cell death. Each type of stress, however, induces specific responses involved in plant acclimation to the particular stress (Kreps et al., 2002; Rizhsky et al., 2004). Therefore, determining the mechanism underlying plant stress tolerance and adaptation is a high priority to ensure plant fitness over a wider range of environmental conditions.

Nitric oxide (NO) is a ubiquitous inter- and intracellular signaling molecule found to be involved in a myriad of cellular functions in plants (Neill et al., 2008b; Mur et al., 2013). Having gained knowledge about its high reactivity and its ambivalent effect, depending on the rate/place of production, there is a need to explore how NO develops all these functions. NO regulates different processes by inducing gene transcription or activating secondary messengers (Besson-Bard et al., 2008; Palmieri et al., 2008; Gaupels et al., 2011). Moreover, NO controls diverse biological processes by directly altering proteins (Martinez-Ruiz et al., 2011). It is able to regulate enzyme activity through covalent post-translational modifications (PTMs) joining metal centers of the proteins and NO tends to affect cysteine and tyrosine residues of the proteins (*S*-nitrosylation and nitration, respectively) changing their activity, location, or aggregation state (Souza et al., 2008; Martinez-Ruiz et al., 2011). Protein *S*-nitrosylation, the incorporation of a nitroso group to a Cys thiol, has been established as a significant route through which NO transmits its global cellular influence, and as a broad-based mechanism for the post-translational regulation of most or all classes of proteins (Stamler et al., 2001; Lindermayr and Durner, 2009; Astier et al., 2011). In this review we will focus on the current state of knowledge regarding *S*-nitrosylation in plants grown under abiotic stress and the elucidation of the function of NO as a signaling mechanism in plant response to environmental modifications.

NO AND ABIOTIC STRESS

Klepper (1979) demonstrated by the end of 1970s that herbicides treated soybean leaves release NO_x (thought to be mainly NO). Then, in the 1990s, new works evidenced plant NO production in response to abiotic stress (Leshem and Haramaty, 1996; Leshem et al., 1998). Notwithstanding, the first confirmations presenting NO as a key signaling molecule were achieved in response to biotic stress (Delledonne et al., 1998; Durner et al., 1998). In parallel a number of reports on exogenous NO effects in plants were shown (Lamattina et al., 2003). From the very beginning the dual effects of NO as a promoter and inhibitor were seen to depend mainly on its concentration (Leshem and Haramaty, 1996; Beligni and Lamattina, 1999). Currently, many examples of the effect of NO donors on plant biology highlight the protective role of NO against abiotic stresses such as salinity, drought, heavy metals, or UV-radiation (Corpas et al., 2006; Tossi et al., 2012a). Special caution needs to be taken with results obtained with NO donors (Murgia et al.,

2004), as each one has specific chemical characteristics that are determinant for the timing of NO release, in addition to features such as the pH of the media, temperature, or light (Ramamurthi and Lewis, 1997; Lamattina et al., 2003). It is therefore difficult to derive physiological conclusions with NO donor effects on plants, as it is hard to measure the real concentration of this molecule in the cell and identify specific targets for each one. Still, the pharmacological approach seems necessary until the mechanism of NO generation in plants can be better defined (Gupta et al., 2011; Mur et al., 2011). Some mutant plants with altered endogenous NO production have been described, involving different NO production pathways or inducing NO production indirectly (Mur et al., 2013). Thus, the use of a combined approach, genetic and pharmacological, would shed light on the functional relevance of NO in response to abiotic stress.

In the past decade, a number of articles have addressed plant endogenous NO production/reduction in a wide range of species, in response to different abiotic stresses (Gould et al., 2003; Corpas et al., 2006; Tossi et al., 2012a). Such studies entail technical problems in assessing the precise location and amount of NO *in planta* (Mur et al., 2013), and the timing and concentration of the stress. It is lacking: are key factors in NO production. Thus, one single stress type could produce contrary effects depending on the application time and strength of the stress. Meanwhile, there are non-technical problems due to the idiosyncrasy of plants, at least seven sources of NO have been described (Gupta et al., 2011), and more than one source could be involved in the response of a certain stress. Contradictory effects might also depend on the species, tissue analyzed, and developmental stage of the plant.

Heavy metals, and specifically Cd provide a clear example of this diversity of NO-timing and effects (see Bartha et al., 2005; Barroso et al., 2006; Rodriguez-Serrano et al., 2006, 2009; Besson-Bard et al., 2009; De Michele et al., 2009). It appears that initial peaks of NO in response to Cd could have to do with signaling functions involved in iron homeostasis and root growth (Besson-Bard et al., 2009).When Cd treatment is in excess (150 μM), NO may be related with programmed cell death (PCD) induction (De Michele et al., 2009), whereas in long-term Cd treatment (50 μM), NO is associated with an induced senescence process stemming from an excess of ROS and ethylene (McCarthy et al., 2001; Romero-Puertas et al., 2002; Rodriguez-Serrano et al., 2009).

Drought is one of the main stresses affecting crop production, and again the role of NO in plant response is not clear (see Leshem and Haramaty, 1996; Magalhaes et al., 2000; Zhao et al., 2001; Garcia-Mata and Lamattina, 2002; Gould et al., 2003; Zhang et al., 2007). An important point is that stomata closure/aperture is essential during drought stress, a process controlled by abscisic acid (ABA), and NO is needed during the ABA-induced stomatal closure of turgid leaves, but there is not such necessity under conditions of rapid dehydration. NO could therefore be involved in the fine tuning of stomata closure in turgid leaves that occurs in response to oscillations in the environment (Garcia-Mata and Lamattina, 2002; Neill et al., 2008a; Wilson et al., 2009).

The literature describes NO responses to other abiotic stresses such as extreme temperatures, salinity, mechanical damage, UV-B, ozone, or herbicides (Gould et al., 2003; Corpas et al., 2006; Neill et al., 2008a; Molassiotis et al., 2010; Tossi et al., 2012b; Sehrawat et al., 2013; Xie et al., 2013). Although much work remains to define the physiological function of this molecule in response to abiotic stress, progress is underway. The NO signaling mechanisms has been building up beginning with transcriptomic analysis (Besson-Bard et al., 2008). NO has also been shown to mediate in different hormone-regulated processes in plants such as salicylic acid (SA), ABA, auxins, ethylene, or DELLAs, and a cross-talk between NO and hormones has been described in response to environmental fluctuations that may involve second messengers such as Ca or kinases (Lamattina et al., 2003; Simontacchi et al., 2013). The existence of a feedback mechanism between NO and ROS has been demonstrated, and ROS/NO balance is an important factor for the fate of the cells, especially in response to abiotic stress in the context of antioxidant systems and ROS production (Neill et al., 2003, 2008b; Rodriguez-Serrano et al., 2009). A further line of study focuses on direct NO-dependent protein regulation, mainly through *S*-nitrosylation and nitration (Astier et al., 2011; Vandelle and Delledonne, 2011).

S-NITROSYLATION UNDER ABIOTIC STRESS

A key feature of NO biology is the PTM of cysteine thiol to form nitrosothiols (*S*-nitrosylation; Stamler et al., 2001). The development of biotin-switch (BST) technology that overcomes the sensitivity of the nitrosothiol group ensures more rapid entry to the world of NO biology. This elegant approach, formulated in the Snyder laboratory, facilitates the identification of *S*-nitrosylated proteins *in situ* as well as *in vitro* (Jaffrey and Snyder, 2001). The BST is currently the most commonly used method, though other promising approaches that are based on the BST have been developed (Seth and Stamler, 2011). Both approaches to detect *S*-nitrosylation have been used in plants grown under abiotic stress, proteome-wide scale and analysis of specific proteins known to be involved in the response of the plant to the mentioned stress (**Table 1**; Astier et al., 2011). Basically, nitrosylating agents (mainly *S*-nitrosoglutathione; GSNO) were used in proteome-wide scale analysis to increase the number of *S*-nitrosylated proteins before facing the proteomic study (Lindermayr et al., 2005; Abat et al., 2008; Abat and Deswal, 2009; Palmieri et al., 2010; Ortega-Galisteo et al., 2012; Begara-Morales et al., 2013; Kato et al., 2013). Other studies have been made in plants grown under stress to pathogen challenge (Romero-Puertas et al., 2008; Maldonado-Alconada et al., 2011) and later on under abiotic stress (Abat and Deswal, 2009; Tanou et al., 2009; Fares et al., 2011; Lin et al., 2012; Camejo et al., 2013). Salinity is the best characterized abiotic stress with regard to *S*-nitrosylation. Forty-nine proteins differentially *S*-nitrosylated were found in *Citrus aurantium* leaves under salt stress. Interestingly, a link between *S*-nitrosylation and oxidative damages (carbonylation) was detected, somehow involved in the prevention of protein loss of function by carbonylation, especially under stress conditions (Tanou et al., 2009). Brief salt stress in *Arabidopsis* cell suspensions showed that NaCl modified the *S*-nitrosylation level of a small proportion of endogenously *S*-nitrosylated proteins (around 10%), suggesting that salt stress induced minor modulations of the *S*-nitrosylation pattern rather than major changes (Wawer et al., 2010; Fares et al., 2011). Interestingly, this lab adapted the method for detection of endogenous

Table 1 | Plant proteins regulated through *S*-nitrosylation in response to abiotic stress.

Plant system	Abiotic stress	NO/GSNOR/SNOs	Number of proteins differentially *S*-nitrosylated	Activity affected by *S*-nitrosylation	Reference
Arabidopsis thaliana leaves	Hypoxia	Increase/–/–	1	AHb1	Perazzolli et al. (2004)
Brassica juncea seedlings	Low temperature (6 h)	–/–/increase	17: 9 up/8 down	Rubisco	Abat and Deswal (2009)
Citrus aurantium leaves	NaCl 150 mM (16 d)	–/–/decrease	49	–	Tanou et al. (2009)
Nicotiana tabacum (BY-2 cells)	NaCl 250 mM (0–60 min)	–/–/–	1	GAPDH*	Wawer et al. (2010)
Arabidopsis thaliana suspension cells	NaCl 100 mM (5 min)	–/–/–	5: 3 up/2 down	–	Fares et al. (2011)
Antiaris toxicaria seeds	Desiccation (6 d)	Increase/–/–	3	APX, GR, DHAR	Bai et al. (2011)
Oryza sativa seedlings (WT vs. *noe 1*)	High light (2 d)	–/–/increase	69**	GAPDH, Trx***	Lin et al. (2012)
Pisum sativum leaves	Cd 50 μM (2 weeks) 2,4-D 23 mM (72 h)	Decrease/decrease /= Decrease/increase/increase	2	CAT, GOX	Ortega-Galisteo et al. (2012)
Pisum sativum mitochondria	NaCl 150 mM (5 d) NaCl 150 mM (14 d)	=/increase/decrease Increase/increase/decrease	9 14	PrxII F	Camejo et al. (2013)

**The enzyme activity was not impaired in vivo following the exposure of BY2 cells to salt or DEA/NO but in vitro.*
***Differentially S-nitrosylated in noe1 vs. WT, all of them under high-light conditions.*
****In this paper it is suggested that S-nitrosylation of GAPDH and TRX in noe1 plants parallels the development of cell death in animal systems.*

S-nitrosylated Cys (Fares et al., 2011). Deeper analysis in isolated organelles could contribute to finding new targets as yet undetected in total extracts; along this lines, a proteomic study was done in mitochondria from pea plants subjected to salt stress (Camejo et al., 2013). A reduction of the *S*-nitrosylation pattern was reported in both short and long-term salt treatment being greater in the latter (Camejo et al., 2013). During salt treatment, proteins from respiratory and photorespiratory pathways and, significantly, antioxidant enzymes changed their *S*-nitrosylation pattern (Fares et al., 2011; Camejo et al., 2013). Changes in the *S*-nitrosoproteome were studied under low temperature as well, with nine spots induced and eight spots reduced differentially identified as plant defense-related, photosynthetic, glycolytic, and signaling-associated mechanisms (Abat and Deswal, 2009). Another noteworthy study looked at the *S*-nitrosoproteome under high-light conditions from wild type (WT) and *noe1* (NO excess1) mutant rice, and showed 48 proteins differentially *S*-nitrosylated in the mutant, 10% related to environmental adaptation and 14% to redox homeostasis (Lin et al., 2012). Indeed, *noe1* mutants have increased H_2O_2 and NO levels and display NO-dependent PCD under high-light conditions. The authors found glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and thioredoxin (Trx) *S*-nitrosylated in *noe1* mutants but not in WT, suggesting a relation of these proteins with the control of light-mediated leaf cell death in rice (Lin et al., 2012), reportedly involved in cell death in animals.

S-nitrosylation pattern of particular proteins involved in a specific stress have been studied in parallel. The first protein identified as undergoing *S*-nitrosylation was hemoglobin AHb1 from *Arabidopsis thaliana*, which reduces NO emission under hypoxic stress through the production of *S*-nitrosohemoglobin (Perazzolli et al., 2004). Then, GAPDH showed a transient increase in its *S*-nitrosylation level in a tobacco cell culture in response to salt stress. However, further analysis is needed to explore the physiological relevance of this change over the treatment period, as its interaction with the osmotic stress-activated protein kinase (NtOSAK) was not affected (Wawer et al., 2010). Antioxidant enzymes from the Asc-Glu cycle, ascorbate peroxidase, glutathione reductase, and dehydroascorbate reductase (APX, GR, and DHAR, respectively) have reduced their *S*-nitrosylated pattern in response to seed desiccation, thereby suggesting a regulation of antioxidant enzyme activities to stabilize H_2O_2 accumulation at an appropriate concentration and increasing seed tolerance to dehydration (Bai et al., 2011). *S*-nitrosylation level of the peroxisomal protein glycolate oxidase decreased under cadmium and 2,4-dichlorophenoxyacetic acid (2,4-D) treatments while no differences were found under 2,4-D in *S*-nitrosylation level of CAT. Also, a reduction of *S*-nitrosylated CAT under Cd treatment was observed but similar to the changes occurred in the total amount of this protein under Cd stress. These results point to a regulation of H_2O_2 level under these stress conditions by NO through the control of ROS sources and antioxidant defenses (Ortega-Galisteo et al., 2012). Additionally, phytochelatins (PCs) with a specific nitrosylation signature were found in *Arabidopsis* cells treated with Cd, suggesting

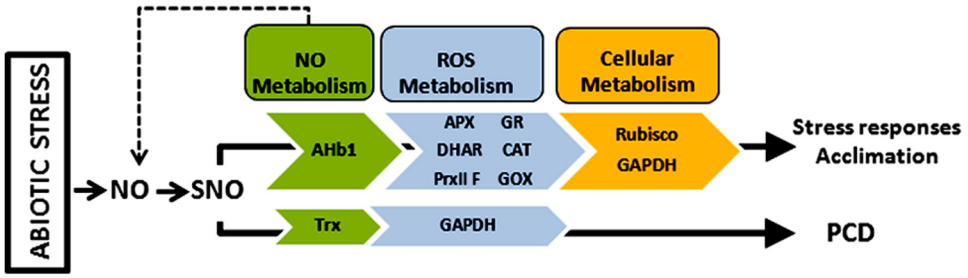

FIGURE 1 | *S*-nitrosylated proteins under abiotic stress. Proteins that change their *S*-nitrosylation pattern and activity in response to abiotic stress are related to NO, ROS, and cellular metabolism. AHb1, haemoglobin 1; APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascobate reductase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GOX, glycolate oxidase; Trx, thioredoxin. It is lacking: PrxII F, peroxiredoxin II F.

an interference with the capacity of PC to chelate the metal (De Michele et al., 2009).

CONCLUSIONS AND PERSPECTIVES

The identification of a number of plant proteins that change their *S*-nitrosylation pattern under abiotic stress is the starting point for the functional and biochemical characterization of *S*-nitrosylation in plants under such pathophysiological conditions (**Figure 1**). While the physiological relevance of *S*-nitrosylation have been shown with various proteins during plant–pathogen interactions (Spoel and Loake, 2011; Yu et al., 2012) this process has been poorly investigated under abiotic stress. Redox and oxygen metabolism-related proteins comprise an interesting group of targets of *S*-nitrosylation under abiotic stress, as these enzymes and the redox state of the cell play a key role in plant responses to environmental changes. A future challenge will be to unravel the NO-dependent control of these proteins, as a fine-tune regulation may exists in the NO/ROS balance to define the fate of the cell, especially under abiotic stress. Likewise important is the determination of NO-dependent interaction with hormones, especially those linked to plant responses to abiotic stress such as ABA (Roychoudhury et al., 2013), given that a link has been found between *S*-nitrosylation and auxins (Terrile et al., 2012). The regulation by *S*-nitrosylation of other protein PTMs that play a crucial role in cellular signaling, including phosphorylation, acetylation, or ubiquitylation, is an intringuing topic (Hess and Stamler, 2012). In this sense, initial data support the idea that *S*-nitrosylation could interfere with protein carbonylation under abiotic stress (Tanou et al., 2012).

Two important open questions regarding NO production can be summed up as "how and where"; especially in response to abiotic stress where the adverse condition is not as localized as in response to pathogens. The regulation of NO levels, and particularly its degradation, would be another topic of debate calling for study. Hemoglobin AHb1 has been shown to detoxify NO during hypoxic stress (Perazzolli et al., 2004), but its involvement in different abiotic stresses is still unknown. GSNO reductase (GSNOR) can control levels of GSNO, indirectly regulating *S*-nitrosylation-dependent signaling, though its participation in abiotic stress must be further explored. Finally, elucidation of the cellular distribution and characterization of nitrosylases and de-nitrosylases that may or may not involve Trx system is a matter essential for our understanding of the full scope of *S*-nitrosylation in plants under physiological and pathophysiological conditions.

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Nitric oxide and phytohormone interactions: current status and perspectives

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Nitric oxide (NO) is currently considered a ubiquitous signal in plant systems, playing significant roles in a wide range of responses to environmental and endogenous cues. During the signaling events leading to these plant responses, NO frequently interacts with plant hormones and other endogenous molecules, at times originating remarkably complex signaling cascades. Accumulating evidence indicates that virtually all major classes of plant hormones may influence, at least to some degree, the endogenous levels of NO. In addition, studies conducted during the induction of diverse plant responses have demonstrated that NO may also affect biosynthesis, catabolism/conjugation, transport, perception, and/or transduction of different phytohormones, such as auxins, gibberellins, cytokinins, abscisic acid, ethylene, salicylic acid, jasmonates, and brassinosteroids. Although still not completely elucidated, the mechanisms underlying the interaction between NO and plant hormones have recently been investigated in a number of species and plant responses. This review specifically focuses on the current knowledge of the mechanisms implicated in NO–phytohormone interactions during the regulation of developmental and metabolic plant events. The modifications triggered by NO on the transcription of genes encoding biosynthetic/degradative enzymes as well as proteins involved in the transport and signal transduction of distinct plant hormones will be contextualized during the control of developmental, metabolic, and defense responses in plants. Moreover, the direct post-translational modification of phytohormone biosynthetic enzymes and receptors through *S*-nitrosylation will also be discussed as a key mechanism for regulating plant physiological responses. Finally, some future perspectives toward a more complete understanding of NO–phytohormone interactions will also be presented and discussed.

Keywords: nitric oxide, plant hormones, auxin, cytokinin, gibberellin, abscisic acid, ethylene, *S*-nitrosylation

INTRODUCTION

As sessile organisms, plants must rely on highly sophisticated signaling mechanisms to adjust their growth, shape, and metabolism with the constant changes in their environment. Playing a key role in this process, plant hormones integrate a multitude of internal and external cues into coordinated metabolic and developmental responses, which, in turn, maximize plant fitness under diverse ontogenetic and environmental contexts. To effectively carry out such critical function, distinct plant hormones intensively interact among themselves and also with other endogenous signaling substances (Santner et al., 2009).

Among these hormone-interacting molecules, the gaseous free radical nitric oxide (NO) has recently gained special interest in the research community given its involvement in a number of signaling cascades controlling plant responses ranging from seed germination to plant senescence (Neill et al., 2003; Wilson et al., 2008; Mur et al., 2012a). Whereas great strides have been made in recent years in understanding the mechanistic relationship between NO and phytohormones in certain physiological responses (Leon and Lozano-Juste, 2011; Terrile et al., 2012; Feng et al., 2013), the exact nature of the interaction between these substances in many developmental, metabolic, and defense events still remains remarkably elusive. In some cases, for instance, it is known that both NO and plant hormones are able to influence a given response, but it is not clear whether they share a common signaling cascade or just modulate the same plant event via parallel, independent signaling pathways.

NO SIGNALING MECHANISMS: WHERE DO WE STAND?

As mentioned by Hancock et al. (2011), characterizing the precise function of NO in a particular signaling event is more difficult than it might appear. Firstly, the particular chemical characteristics of NO inexorably imply peculiar mechanisms for "sensing" the presence and levels of this signaling molecule. Instead of a unique or very few receptors, NO likely interacts with a wide range of target proteins via direct modification of protein structure (**Figure 1**). Through these chemical modifications of target proteins, NO may trigger changes in their activities and cellular functions, ultimately leading to the transduction of the NO message into plant responses.

Among the biologically relevant NO-dependent post-translational modifications (PTMs), the covalent modification of cysteine residues through a processes known as *S*-nitrosylation (**Figure 1A**) has been emerging as a critically important

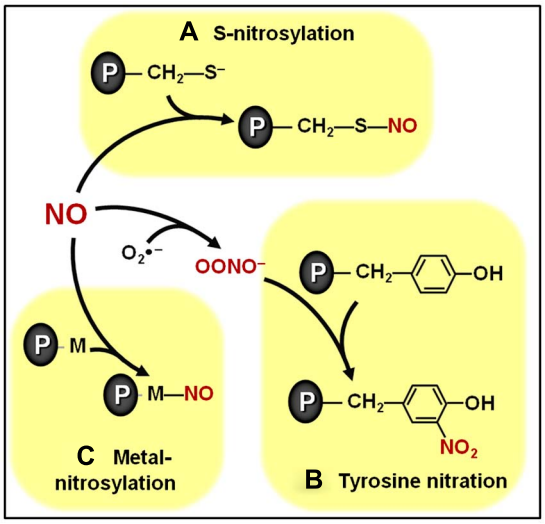

FIGURE 1 | Overview of biologically relevant NO-dependent post-translational modifications (PTMs). (A) *S*-nitrosylation of cysteine residues. **(B)** Tyrosine nitration. **(C)** Metal nitrosylation. Proteins are represented with gray ovals and "P" letters.

mechanism intermediating NO signal transduction in plants (Lindermayr et al., 2005; Astier et al., 2011, 2012). This specific, reversible and regulated NO-dependent PTM has been implicated as potentially controlling the function of components of plant processes as diverse as cellular architecture, photosynthesis, genetic information processing, protection against oxidative stress, defense responses to biotic and abiotic stresses, hormonal signaling, among others (Lindermayr et al., 2005; Romero-Puertas et al., 2008; Astier et al., 2011, 2012; Astier and Lindermayr, 2012). Currently, some of the best characterized examples of *S*-nitrosylation in plant systems include the modulation of phytohormone biosynthetic enzymes (Lindermayr et al., 2006), receptors (Terrile et al., 2012), and signal transduction proteins (Feng et al., 2013), which will be discussed in more detail later in this review. The specificity of this NO-triggered PTM is essentially based on the fact that only cysteine residues surrounded by particular neighboring amino acids seem to be the target of *S*-nitrosylation (Astier et al., 2011; Kovacs and Lindermayr, 2013).

A second physiologically relevant NO-dependent PTM depends on the reaction between NO and reactive oxygen species (ROS), such as superoxide (O_2^-), resulting in the production of NO-derived species, such as peroxynitrite ($ONOO^-$), which, in turn, can covalently modify tyrosine residues through a process known as tyrosine nitration (**Figure 1B**; Astier and Lindermayr, 2012). Initially considered an irreversible process, tyrosine denitration is now believed to occur either enzymatically or non-enzymatically (Abello et al., 2009; Vandelle and Delledonne, 2011; Astier and Lindermayr, 2012). Reinforcing such reversibility in tyrosine nitration, transient, rather than permanent, changes in the abundance of nitrated proteins have already been reported in the literature (Cecconi et al., 2009). More research is required to better define the biological relevance of this NO-dependent protein modification in plants, which apparently may target proteins involved in many basic cellular processes, such as photosynthesis, respiration and nitrogen metabolism (Cecconi et al., 2009; Chaki et al., 2009b; Lozano-Juste et al., 2011; Tanou et al., 2012)

In addition to *S*-nitrosylation and tyrosine nitration, a third important NO-dependent PTM involves the binding of NO to transition metal centers of metalloproteins in a process known as metal nitrosylation (**Figure 1C**). Currently, one of the best characterized examples of metal nitrosylation is the activation of soluble guanylate cyclase (sGC) in animal systems (Ignarro et al., 1999). In plants, although cyclic guanosine monophosphate (cGMP) has already been reported as an important intermediate in several NO-induced processes, including root development, mitochondrial respiration, nodule functioning, and defense responses (Durner et al., 1998; Pagnussat et al., 2003; Ederli et al., 2008; Keyster et al., 2010; Wang et al., 2010), more studies are still required to clarify whether metal nitrosylation also regulates plant sGC.

Regardless of the specific type of NO-triggered PTM considered, these chemical modifications may represent a central mechanism through which NO impacts signaling networks responsible for controlling plant development and metabolism. In responses regulated by plant hormones, for instance, these PTMs might facilitate the influence of NO on hormonal production and/or action via three distinct but non-exclusive mechanisms. The first mechanism implicates NO-dependent chemical modifications of proteins (e.g., transcription factors, regulatory proteins, and channels) whose functions may not be directly implicated in plant hormone metabolism, distribution, or signaling but, instead, may influence the abundance of other proteins more intimately implicated in such specific roles (**Figure 2A**). In contrast, a second and more direct way involves the NO-triggered PTM of proteins directly associated with the production, degradation, conjugation, transport, perception, or signaling transduction of plant hormones (**Figure 2B**). For example, in the first mechanism, NO may chemically modify a transcription factor that stimulates the production of a hypothetical enzyme responsible for hormone degradation, whereas in the second mechanism, NO would directly interact and modify the activity, stability, and/or cellular localization of this degradative enzyme (**Figures 2A,B**). A third possibility recently described in the literature involves the direct chemical reaction between NO-derivates (e.g., peroxynitrite) and certain hormonal species (e.g., zeatin), rendering products with altered biological activity (**Figure 2C**). Specific examples of all three of these mechanisms of NO–phytohormone interaction will be provided and discussed later in this review.

NO SIGNALING SPECIFICITY: HOW CAN SUCH A SMALL MOLECULE CONTROL SO MANY PROCESSES?

Considering that a massive number of proteins, peptides, and other molecules may undergo changes in their structure and activity via direct NO-dependent chemical modifications (Astier et al., 2011, 2012; Astier and Lindermayr, 2012) and an equivalent amount of genes may have their transcription levels influenced by NO (Polverari et al., 2003; Parani et al., 2004; Grun et al., 2006; Besson-Bard et al., 2009), one pertinent question that arises is how NO signals can confer sufficient specificity to trigger coordinated

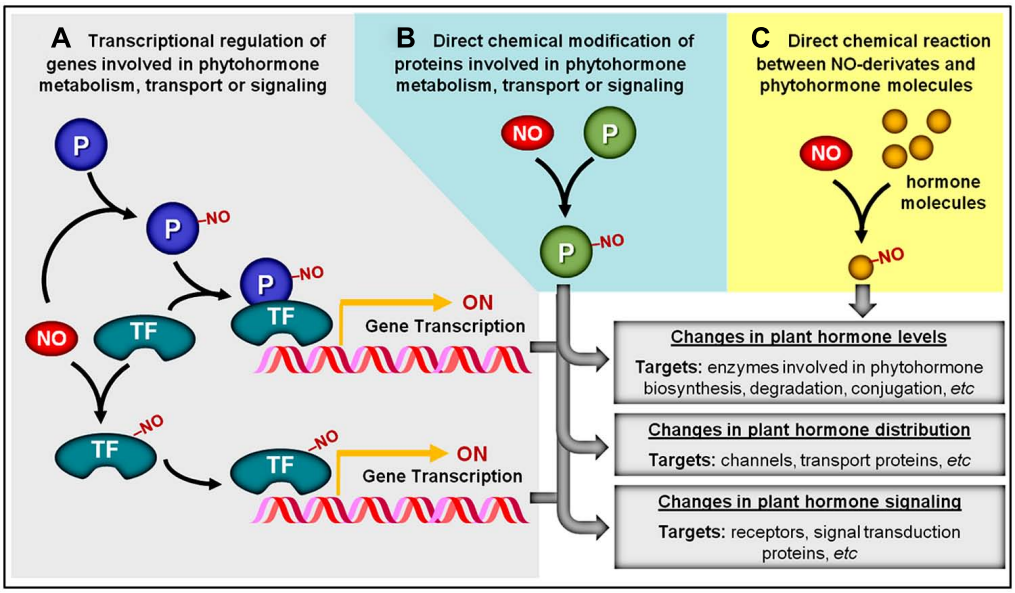

FIGURE 2 | Overview of potential NO–phytohormone interaction mechanisms. (A) By chemically modifying transcription factors (TF) and other proteins (P), NO may influence the transcription level of genes involved in phytohormone metabolism, transport, or signal transduction. **(B)** NO may post-translationally modify proteins (P) directly involved in the production, distribution, or signaling of plant hormones. **(C)** NO or NO-derived reactive species might also chemically react with certain plant hormonal species, rendering products with altered biological activity. NO-dependent chemical modifications are represented by "–NO."

downstream effects. Although answering this question involves a certain degree of speculation at this point in the research of NO signaling in plants, aspects such as spatial and temporal signaling compartmentation and a precise control of NO biosynthesis and removal might possibly be key to explaining how a molecule as small as NO might be responsible for controlling so many plant responses.

As is the case with cytosolic Ca^{2+}, a strict temporal and spatial regulation of NO levels inside each plant cell might be essential for delivering sufficiently specific NO signals. The transient generation of "NO hot-spots," in particular plant cell compartments, could lead to compartmentalized protein modifications (Neill et al., 2008b), and, consequently, the NO signals may be sensed by a specific group of proteins responsible for a particular set of cellular functions. A possible mechanism for assuring such localized action of NO could be the existence of macromolecular modules including all major NO signaling components (e.g., NO biosynthetic enzymes, NO removal enzymes, and targets of NO-dependent PTMs). Although such macromolecular complexes have not yet been described in plants, recent models for NO-mediated stress signaling in animal systems suggest, for instance, that the control of certain membrane calcium channels via reversible *S*-nitrosylation is facilitated by the close proximity of these channels to the NO-generating enzyme (Stamler and Meissner, 2001). Therefore, in this case, instead of a global change in cellular NO levels, the transient production of this signaling molecule at particular regions of the animal cell may control the activity of nearby target proteins via reversible *S*-nitrosylation (Martinez-Ruiz et al., 2013). As an ultimate consequence, such compartmentation and fine-tuned dynamics of NO production could minimize a certain spatial promiscuity in terms of concomitant occurrence of NO, NO-derivates, and their target proteins.

A relevant bottleneck for advances in the evaluation of the possible existence of such NO signaling macromolecular modules in plants is the still incipient characterization not only of the targets of NO-dependent PTMs but also of the biosynthetic and removal machinery responsible for controlling NO levels inside the plant cell compartments. Interestingly, though, compartmentalized production of NO has already been reported in plant cells. Foissner et al. (2000), for example, reported that after challenging epidermal tobacco cells with the elicitor cryptogein, NO accumulation first appeared in the plastids and subsequently in other cell compartments, such as the nucleus and the cytoplasm.

NO PRODUCTION AND REMOVAL: WHY SO MANY PATHWAYS IN PLANTS?

Placing NO as an element of a given signaling cascade necessarily implies that changes in its levels or cellular localization might occur during the course of the signaling event. Therefore, characterizing the specific changes in the NO biosynthetic and degradation mechanisms responsible for delivering adequate concentrations of this molecule at the right time and place seems a logical step in any research interested in discriminating the actual role of NO during the regulation of specific plant responses. However, the relevance of the different origins of NO in plants is still poorly understood; as a consequence, controversy and ambiguity are still frequently found in the current literature (Kaiser and Planchet, 2006; Gupta et al., 2011).

Besides the non-enzymatic NO production, which is believed to occur only under very specific conditions (Bethke et al., 2004),

so far, seven potential enzymatic sources of NO have been identified in plants (**Figure 3A**; Gupta et al., 2011). Among them, nitrate reductase (NR) and NO synthase-like (NOS-like) activities are currently considered as the most likely candidates for the production of NO under physiologically relevant conditions (Neill et al., 2008b; Mur et al., 2012a). Since the discovery that plant NR could produce NO both under *in vitro* and *in vivo* conditions (Harper, 1981), a great deal of evidence has indicated this enzyme as one of the major plant biosynthetic sources of NO (Rockel et al., 2002; Meyer et al., 2005; Kaiser et al., 2010). Supporting this view, pharmacological and genetic approaches in different plant species, organs, tissues, and experimental conditions have revealed that NR inhibition frequently results in decreased NO production (Planchet and Kaiser, 2006; Oliveira et al., 2009; Freschi et al., 2010; Kolbert et al., 2010; Lombardo and Lamattina, 2012). On the other hand, the existence of NOS-like activity in plants is exclusively supported by biochemical and pharmacological evidence since a canonical NOS gene or a mutant deficient in NOS-like-dependent NO production has not been identified in higher plants yet (Corpas et al., 2006; Gupta et al., 2011; Mur et al., 2012a). Thus far, the organism more closely related to higher plants in which such a gene was described is the photosynthetic microalgae *Ostreococcus tauri* (Foresi et al., 2010; Correa-Aragunde et al., 2013), which belongs to a basal branch of the flowering plant evolutionary tree.

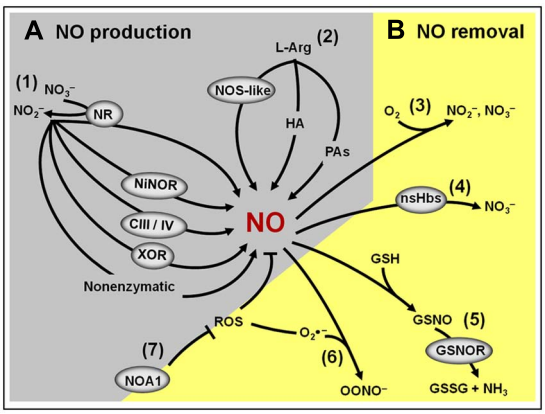

FIGURE 3 | Overview of the NO production and removal mechanisms in plants. (A) Main components of the NO biosynthetic machinery: (1) Nitrite-dependent NO production in plants includes a non-enzymatic pathway and several enzymatic pathways involving the action of cytosolic and plasma membrane nitrate reductases (NR), nitrite–NO reductase (NiNOR), mitochondrial electron transport chain (CIII/IV) and xanthine oxidoreductase (XOR). (2) L-Arginine-dependent NO production pathway involves a non-identified nitric oxide synthase (NOS)-like enzyme and two still poorly characterized pathways using hydroxylamine (HA) or polyamines (PAs) as substrates. **(B)** Main components of the NO removal machinery: (3) the reaction of NO with molecular oxygen leads to the spontaneous production of nitrite and nitrate. (4) NO can react with non-symbiotic hemoglobins (nsHbs) resulting in nitrate formation. (5) Alternatively, NO may react with reduced glutathione (GSH) to form *S*-nitrosoglutathione (GSNO), which, in turn, can be converted into oxidized GSSG and ammonia by the action of GSNO reductase (GSNOR). (6) NO can also react with superoxide (O_2^-), resulting in the formation of peroxynitrite ($OONO^-$). (7) By influencing the production of reactive oxygen species (ROS), NO-ASSOCIATED 1 (NOA1) protein indirectly impacts NO levels in plants.

In 2003, studies revealed that *NO Associated1* (*AtNOA1*), formerly described as *AtNOS1* (Guo et al., 2003; Guo and Crawford, 2005; Zemojtel et al., 2006), also significantly influences NO generation in *Arabidopsis*. However, according with the latest consensus in the literature, *AtNOA1* encodes a chloroplast-localized cGTPase probably involved in ribosome assembly and subsequent mRNA translation to proteins in this organelle (Flores-Perez et al., 2008; Moreau et al., 2008). Therefore, the reduced NO production observed in *noa1* mutants is currently interpreted as an indirect outcome of disturbances in chloroplast metabolism due to the lack of *AtNOA1* function (Zemojtel et al., 2006; Gas et al., 2009). More recently, this mutant was crossed with the NR-deficient *nia1-nia2* mutant of *Arabidopsis*, generating a triple mutant (*nia1,2noa1,2*), which presented no detectable NO production and a range of physiological and developmental disturbances (Lozano-Juste and Leon, 2010a), thereby reinforcing the physiological importance of these pathways for determining the endogenous NO levels in plants.

Another important and frequently neglected aspect that may influence NO metabolism and signaling in plants is the presence of efficient mechanisms for removing the NO signal from a particular cell type or compartment as soon as it is no longer required. Besides the inherent chemical instability of NO in the presence of oxygen, this molecule might also be removed from plant tissues by several biochemical mechanisms (**Figure 3B**; Neill et al., 2008b; Mur et al., 2012a). Firstly, NO can be removed by reacting with ROS, such as superoxide anions, generating peroxynitrite. Secondly, NO may interact with plant proteins, such as non-symbiotic hemoglobins (nsHbs), which facilitates its oxidation to nitrate (Perazzolli et al., 2006). Finally, NO might also react with thiol proteins and peptides, resulting in the formation of *S*-nitrosothiols. In plant tissues, one of the most abundant low-molecular-mass *S*-nitrosothiols is the intracellular antioxidant glutathione, which may react with NO or with the NO-derivate N_2O_3, generating *S*-nitrosoglutathione (GSNO; Neill et al., 2008b; Mur et al., 2012a). The GSNO formed can spontaneously liberate NO or be metabolized by the enzyme *S*-nitrosoglutathione reductase (GSNOR), originating oxidized glutathione (GSSG) and NH_3 (Barroso et al., 2006; Corpas et al., 2008b; Leterrier et al., 2011). Besides being an intracellular NO reservoir, GSNO may also be transported between cells, possibly playing a critical role as a vehicle of the NO signal throughout the plant body (Corpas et al., 2013).

NO–PHYTOHORMONE INTERACTIONS: GENERAL MECHANISMS AND IMPLICATIONS

Before exploring the general mechanisms underlying the interactions between NO and phytohormones, it is worth mentioning that a great diversity of methodological approaches, experimental designs, and plant models have been used in NO research, which sometimes makes it difficult to directly compare the literature data. In terms of methodological approaches, for instance, a considerable variety of analytical techniques have been employed to determine NO levels in plant systems, including the Griess and the hemoglobin assays, electron spin resonance, laser-based photoacoustic detection, ozone-based chemiluminescence, and various fluorescent probes (reviewed by Vitecek et al., 2008 and Mur et al.,

2011). As expected, these different methods provide distinct information. For example, it is always a challenge to compare results obtained by gas-phase NO detection techniques (e.g., chemiluminescence or laser photoacoustic) with fluorescent methods for *in situ* NO detection since these two groups of techniques differ greatly in their specificity, spatial resolution, and capacity to indicate the actual concentration of NO inside the target cells (Mur et al., 2011). Furthermore, evaluations of NO levels under the same experimental conditions by two or more independent methods, although recommended (Mur et al., 2012a; Gupta and Igamberdiev, 2013), are rarely carried out (Besson-Bard et al., 2008).

Besides measuring NO itself, alternatives to access NO and other RNS signaling inside the cells, such as the abundance of NO-triggered chemical modifications on proteins and peptides, have also recently drawn increasing attention of the plant research community, providing, in some cases, spectacularly relevant information. A number of technical options are currently available for such proposes, including the determination of *S*-nitrosothiol levels in plant extracts by reductive gas-phase chemiluminescence (Valderrama et al., 2007; Corpas et al., 2008b; Lee et al., 2008; Chaki et al., 2009a), immunolocalization of particular *S*-nitrosylated thiols or proteins (Barroso et al., 2006; Valderrama et al., 2007; Corpas et al., 2008a; Chaki et al., 2009a; Leterrier et al., 2011) or even proteomic profiling of proteins chemically modified by NO or NO-derivates (Lindermayr et al., 2005; Chaki et al., 2009b; Astier et al., 2011; Kovacs and Lindermayr, 2013), which, among other aspects, may facilitate the identification of the specific targets of NO-dependent PTMs in distinct plant responses.

Another relevant aspect to be considered in NO–phytohormone interaction studies is that the simple observation of changes in NO levels triggered by exogenous plant hormones does not necessarily imply a straightforward relationship between NO and the hormonal stimulus. Firstly, the exogenous application of a signaling substance might potentially induce global, unspecific changes in plant biochemistry, metabolism, and development. Secondly, modifications in NO levels might sometimes result from excessive levels of exogenous hormones; therefore, whenever possible, the actual concentration of particular phytohormone species inside the plant cells and tissues should be determined following the supplementation with these substances. Finally, some plant hormones may affect the biosynthesis and signaling of others (Santner et al., 2009); consequently, the establishment of a direct correlation between the pharmacological effect of a specific plant hormone on a given cellular response is not always an easy task. To overcome such a lack of specificity and potentially artificial effects, the use of transgenic and mutant plants with altered production, degradation or signaling of particular hormonal classes as well as a detailed characterization of several elements involved in phytohormone and NO metabolisms and signaling transduction have proven to be a powerful strategy for accessing the mechanistic relationship between these substances (Desikan et al., 2002; Leon and Lozano-Juste, 2011; Terrile et al., 2012).

Despite these methodological disparities and the limited literature information currently available, there is virtually no doubt that NO and phytohormones interact at multiple, diversified levels. Depending on the signaling cascade, NO has been demonstrated to act either upstream or downstream of plant hormones (Hancock et al., 2011; Simontacchi et al., 2013). Obviously, placing NO downstream of the hormonal stimuli in a signaling route necessarily means that the NO biosynthetic, degradation, conjugation, or deconjugation machinery may be affected at certain point between the perception of hormonal stimulus and the induction of the plant response. Therefore, the time period between the hormonal message input and the detection of changes in endogenous NO levels represents valuable information. In some cases, lag phases compatible with changes in the transcripts level or protein abundance of NO-synthesizing or removal enzymes have been reported (Pagnussat et al., 2002; Freschi et al., 2010). However, under some particular circumstances, the lag phase observed between the application of plant hormones and the rise in NO endogenous levels has been shown to be of just few minutes (Tun et al., 2001; Garcia-Mata and Lamattina, 2002; Huang et al., 2004; Tun et al., 2006; Sun et al., 2010), which indicates that the post-translational regulation of proteins involved in NO metabolism rather than their *de novo* synthesis might sometimes be implicated.

When acting upstream of phytohormones, NO seems able to modulate elements controlling either the plant hormone levels (e.g., biosynthetic, degradation, and conjugation enzymes), distribution (e.g., transport proteins) or signaling (e.g., receptors and signal transduction proteins). This modulation has been shown to occur either at the transcriptional (Bethke et al., 2007; Liu et al., 2009; Manjunatha et al., 2010; Xu et al., 2010; Leon and Lozano-Juste, 2011) or post-translational levels (Lindermayr et al., 2006; Terrile et al., 2012; Feng et al., 2013); however, some post-transcriptional or even translational regulation of hormone-related proteins by NO, although not yet demonstrated, cannot be ruled out.

Based on the basic information provided thus far, the current state-of-the-art of the interplay between NO and each one of the major classes of plant hormones [i.e., auxins, cytokinins, gibberellins (GAs), abscisic acid (ABA), and ethylene] will now be discussed. Although discussed here in an isolated manner, it is important to keep in mind that very frequently, if not always, plant hormones intensively interact with each other during the induction and establishment of plant responses. However, future studies will still be required to mechanistically explain exactly how distinct plant hormones concomitantly interact with NO to regulate specific plant events.

NO AND AUXINS INTERACTIONS

Synergistic effects of auxin and NO have been observed during the regulation of a series of plant responses, including root organogenesis (Pagnussat et al., 2002, 2003, 2004; Lanteri et al., 2006), gravitropic responses (Hu et al., 2005), root nodule formation (Pii et al., 2007), root responses to iron deficiency (Chen et al., 2010), activation of cell division and embryogenic cell formation (Ötvös et al., 2005), NR activity stimulation (Du et al., 2008), among others. In virtually all of these cases, NO was identified to function downstream of auxins, apparently through linear signaling pathways. Increased NO production has frequently been observed after exogenous auxin application (Pagnussat et al., 2002; Correa-Aragunde et al., 2004; Hu et al., 2005; Lombardo et al., 2006) or in

auxin overproducer mutants (Chen et al., 2010), being especially evident in plant tissues or cells undergoing auxin-dependent physiological responses. On the other hand, no or weak stimulation in NO production by auxins has been reported in some particular experimental conditions or cell types (Tun et al., 2001; Guo et al., 2003), suggesting that the auxin-dependent NO production may occur exclusively under specific temporal and spatial contexts (Hu et al., 2005).

Currently, most of the reports on NO and auxin interaction are focused on plant root responses, with relatively little information available on the crosstalk between these two signaling molecules in shoot or reproductive tissues. During the last decade, detailed information about the interaction between NO and auxin during root growth and development was provided by a series of studies conducted by Lamattina and colleagues, including the interplay between these molecules during adventitious roots formation (Pagnussat et al., 2002, 2003, 2004), lateral root development (Correa-Aragunde et al., 2004), and root hair initiation and elongation (Lombardo et al., 2006). In almost all of these studies, the removal of NO by scavengers significantly decreased typical auxin-dependent root responses, such as the activation of mitogen-activated protein kinases (MAPKs) during the adventitious root formation (Pagnussat et al., 2004) and induction of cell cycle genes during lateral root formation (Correa-Aragunde et al., 2006).

Also focusing on root tissues responses, Chen et al. (2010) identified a direct correlation between auxin availability, root NO levels and the expression of iron acquisition genes and other Fe deficiency-associated stress responses, providing further support for the action of NO as a downstream element in the auxin signaling pathway. Similarly, a clear spatial correlation was also observed between the asymmetric auxin distribution and the endogenous NO localization during the gravitropic bending in soybean roots (Hu et al., 2005) and during indeterminate nodule formation in roots of *Medicago* species infected by auxin-overproducing rhizobia (Pii et al., 2007).

A possible role for NR as the major biosynthetic source of the auxin-induced NO production during some plant root responses has been suggested (Kolbert and Erdei, 2008). Kolbert et al. (2008), for instance, reported that the NO production during the auxin-induced lateral root development in *Arabidopsis* requires NR activity since the NR-deficient double mutant *nia1,nia2* failed to increase NO generation in response to exogenous auxin, whereas no evidence for an involvement of NOS in this response was observed. NR-dependent NO production was also shown to be crucially important for the adequate vesicle trafficking during root hair formation because exogenous NO application completely restored the abnormal vesicle formation and trafficking as well as root hair growth in the *nia1,nia2 Arabidopsis* mutant (Lombardo and Lamattina, 2012). In a few cases, however, such as during the auxin-regulated NO generation under Fe deficiency and during the gravitropic bending in soybean roots, evidence indicates the involvement of not only NR but also NOS and/or NOA1 in the auxin-induced NO generation (Hu et al., 2005; Chen et al., 2010).

Considering that many of these root responses, including root hair formation and lateral root development, respond to both auxins and nitrate supply, NR-dependent NO generation might be a key integrator of exogenous and endogenous cues leading to the control of plant root biology. Although the precise mechanism through which auxin trigger NR-dependent NO generation has still not been fully characterized, literature data indicate a promotive effect of this plant hormone on NR protein, activity and gene transcription (Vuylsteker et al., 1997; Du et al., 2008).

Besides these impacts of auxin on NO production, recent studies have demonstrated that NO might also modulate auxin metabolism, transport, and signaling. For example, NO has been demonstrated to enhance root indole-3-acetic acid (IAA) levels in cadmium-treated *Medicago truncatula* seedlings by reducing its degradation via IAA oxidase activity (**Figure 4**), thereby positively impacting auxin equilibrium and ameliorating cadmium toxicity (Xu et al., 2010). In addition, pharmacological treatments and NO-overproducing mutants indicated that, at high concentrations, NO inhibits acropetal auxin transport in *Arabidopsis* roots by reducing the abundance of the auxin efflux protein PIN-FORMED 1 (PIN1) via a proteasome-independent post-transcriptional mechanism (Fernández-Marcos et al., 2011). This NO-dependent decrease in PIN1 protein levels and consequent disturbance in root auxin transport resulted in severe reductions in root meristem size and activity in primary roots due to a reduction in cell division and a promotion in cell differentiation, compromising the root apical meristem maintenance and primary root growth (Fernández-Marcos et al., 2011).

Finally, a direct influence of NO on auxin perception and signal transduction has also been suggested based on the recent demonstration that the auxin receptor protein TIR1 (TRANSPORT INHIBITOR RESPONSE 1) undergoes *S*-nitrosylation at two particular cysteine residues (cys-140 and cys-480) (Terrile et al., 2012). This *S*-nitrosylation of TIR1 seems to promote its interaction with AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins, which are transcriptional repressors of genes associated with auxin responses (**Figure 4**). Being part of an E3 ubiquitin ligase complex, TIR1 marks AUX/IAA proteins to proteasome degradation, de-repressing the expression of auxin-dependent genes. Therefore, as a result, the increased TIR1–AUX/IAA interaction caused by TIR1 *S*-nitrosylation may facilitate AUX/IAA degradation via proteasome and subsequently promote auxin-dependent gene expression (Terrile et al., 2012). A possible impact of *S*-nitrosylation on the capacity of TIR1 to bind auxin could also be a possible outcome of this NO-dependent PTM, but further investigations are still required on this subject.

Furthermore, evidence indicates that nsHbs might also influence and modify the auxin signaling and action site by modulating the endogenous NO levels. Hunt et al. (2002), for example, detected a drastic modification in auxin-regulated root morphology and development in transgenic lines of *Arabidopsis* overexpressing class 1 nsHb, which could be interpreted as the result of changes in the content and/or bioactivity of NO in these plants.

NO AND CYTOKININS INTERACTIONS

During the last few years, accumulating evidence has indicated complex and multilevel interactions between NO and cytokinins. Both synergistic and antagonistic interactions between NO and cytokinins have been described depending on the physiological

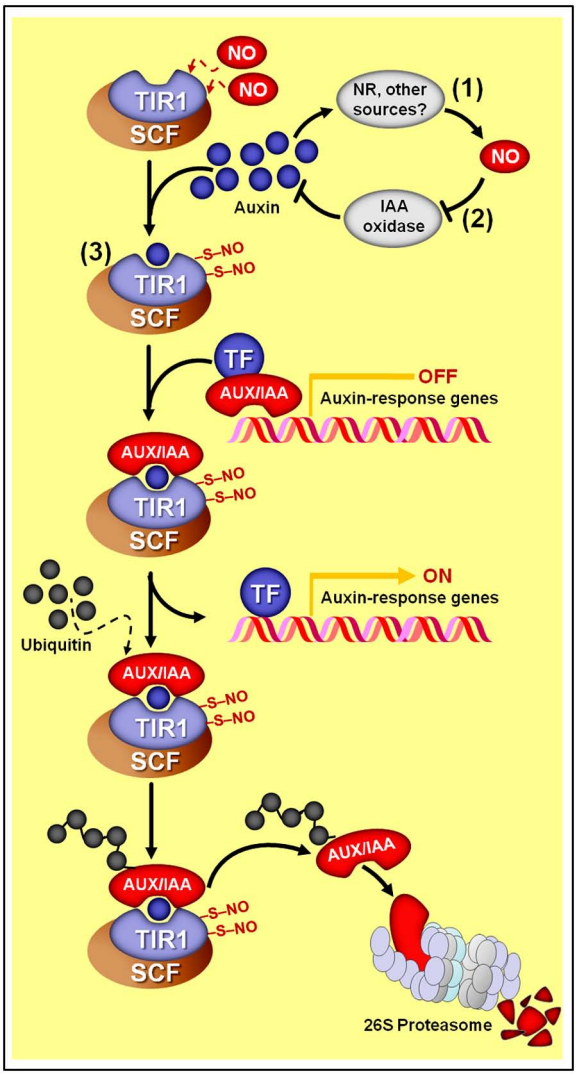

FIGURE 4 | Schematic representation of NO–auxin synergist interactions. (1) Auxins stimulate NO production in several plant materials and experimental conditions. In most cases, nitrate reductase (NR) seems to be the main biosynthetic source of auxin-induced NO production. (2) In *M. truncatula* roots, NO promotes auxin accumulation by repressing its degradation via IAA-oxidase. (3) In *Arabidopsis*, NO might also positively impact auxin signaling since the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1) may undergo *S*-nitrosylation at cys-140 and cys-480, which promotes its interaction with AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins. Subsequently, TIR1 marks AUX/IAA proteins to degradation through SCF-26S proteasome-mediated proteolysis, thereby de-repressing the transcription of auxin-regulated genes. Protein *S*-nitrosylation is represented by "–S–NO."

response, plant species and experimental approach. Evidence implying a possible participation of NO in cytokinin signal transduction was first obtained during the accumulation of the red pigment betalaine in *Amaranthus caudatus* seedlings, which was shown to positively respond not only to cytokinins but also to NO gas or donors (Scherer and Holk, 2000). Since then, a number of studies have reported rapid and dose-dependent increases in NO production triggered by μM concentrations of cytokinins in both plant cell cultures (Tun et al., 2001; Carimi et al., 2005) and intact seedlings (Tun et al., 2008; Shen et al., 2012). In *Arabidopsis* seedlings, for instance, zeatin triggered increases in NO production within 3 min via a biosynthetic mechanism sensitive to arginine analogs and apparently independent of NR activity (Tun et al., 2008). However, other evidence revealed unchanged or even lower NO levels after cytokinin treatments or in mutant or transgenic plants with increased cytokinin production (Xiao-Ping and Xi-Gui, 2006; Romanov et al., 2008; Liu et al., 2013). Moreover, no obvious influence of exogenous application or depletion of NO has been observed on some early signaling events leading to the induction of primary cytokinin responses, such as the activation of cytokinin-responsive *Arabidopsis response regulator* (*ARR*)*5* promoter in seedlings (Romanov et al., 2008).

Examples of synergistic interaction between cytokinins and NO include the control of leaf senescence (Mishina et al., 2007), programmed cell death (PCD; Carimi et al., 2005), photosynthesis adaptability to drought stress (Shao et al., 2010), cell division, and differentiation (Shen et al., 2012), among others. Studies of the integrated influence of NO and cytokinins on plant senescence program have demonstrated that natural, dark- or dehydration-induced leaf senescence can be minimized by exogenous NO application (Cheng et al., 2002; Mishina et al., 2007). In addition, mutant or transgenic plants exhibiting decreased NO levels usually display precocious senescence in detached leaves and intact plants (Guo and Crawford, 2005; Mishina et al., 2007), which can sometimes be alleviated by exogenous cytokinin supplementation (Mishina et al., 2007). Although still limited in terms of current commercial application, this antisenescence trait of NO and cytokinins has been proven to extend post-harvest life of agronomically relevant fruits and vegetables (Leshem and Wills, 1998; Leshem et al., 1998; Leshem et al., 2001).

Further indicating a protective and antisenescence role of NO and cytokinins, Shao et al. (2010) reported increased NO levels during the cytokinin-induced photosynthetic adaptability to drought stress and described a good correlation between NO production and NR activity during this adaptive plant response to water limitation. In contrast, however, NOS-like-dependent increases in NO generation have been suggested to act as an intermediate during the acceleration of cell apoptosis induced by high cytokinin dosages since cell death was alleviated when cytokinins were supplied along with NOS inhibitors or NO scavengers to *Arabidopsis* cell cultures (Carimi et al., 2005).

The interaction between cytokinins and NO during the regulation of plant cell division has also been recently studied in more detail. Among other evidence, NO deficiency caused either by loss of the gene *NOA1* or due to NO scavenger treatments was demonstrated to result in severe inhibition of cytokinin-induced transcriptional activation of the cell cycle gene *CYCD3;1* (*CYCLIN-D3;1*) and the subsequent callus initiation from somatic plant tissues, implying that NO may act downstream of cytokinins in the control of plant cell mitotic cycles (Shen et al., 2012). In this study, roots of *Atnoa1* mutant were described as severely impaired in cytokinin-induced NO production and less sensitive to cytokinins than wild-type (WT) ones (Shen et al., 2012).

Contrary to the above described synergistic relationships between NO and cytokinins, literature data have also suggested

an opposite interaction between these signaling molecules in some plant responses (Xiao-Ping and Xi-Gui, 2006). Studies conducted on epidermal strips of *Vicia faba* indicated that exogenous cytokinins efficiently reduced NO generation in guard cells exposed to the NO donor sodium nitroprusside (SNP) as well as promoted stomata reopening under dark condition due to the abolishment of the dark-induced increases in endogenous NO, which was interpreted as evidence of a potential scavenging action of cytokinins on the NO produced under these situations (Xiao-Ping and Xi-Gui, 2006).

Consistent with these results, Wilhelmová et al. (2006) also observed a negative correlation between endogenous cytokinin and NO levels in transgenic tobacco plants with either increased or decreased cytokinin levels. More recently, Liu et al. (2013) reported that cytokinins might intimately participate in NO catabolism since some cytokinin species, such as zeatin, can chemically react with peroxynitrite, leading to the production of cytokinin derivates with virtually no biological activity (**Figure 5**). Moreover, these authors verified that exogenous zeatin alleviates the severity of the phenotypes attributed to excessive NO levels in the *Arabidopsis* NO-overproducer *nox1* (*nitric oxide overexpression 1*) mutant, and this same ameliorative effect was observed when *nox1* plants were crossed with a cytokinin-overproducing mutant (Liu et al., 2013). Based on these biological and chemical data, Liu et al. (2013) postulated that these two signaling molecules (NO and cytokinins) might interact by modulating each other's homeostatic levels and bioactivity (**Figure 5**). Such peculiar mechanism of interaction between cytokinins and NO, in which one of the substances directly interferes with the levels of another simply by a chemical combination of two molecules (**Figure 2C**), is quite different from the interaction at biosynthetic or signaling levels usually observed for other NO–phytohormone crosstalks (**Figures 2A,B**) and certainly deserves further attention.

Additionally, strong evidence indicating a direct impact of NO on the cytokinin signaling pathway has recently been uncovered (Feng et al., 2013). Besides corroborating previous observations that *Arabidopsis* mutant lines with excessive NO levels display more limited responsiveness to cytokinins, Feng et al. (2013) revealed that the phosphorelay mechanism central to the signaling transduction of this hormonal class can be severely impaired by the *S*-nitrosylation of a particular cysteine residue (cys 115) of the HISTIDINE PHOSPHOTRANSFER PROTEIN 1 (AHP1), hindering the transfer of phosphoryl groups from cytokinin receptors to AHP1 and subsequently to response regulators (ARRs; **Figure 5**). Confirming the importance of this NO-dependent post-translational protein modification for the cytokinin signal transduction, these authors have demonstrated that non-nitrosylatable mutation of AHP1 consistently relieved the inhibitory effect of NO on cytokinin responses whereas a nitrosomimetic mutation of this protein severely compromised cytokinin responses (Feng et al., 2013).

An additional, less direct way through which cytokinins might modulate NO levels in plant systems seems to rely on the regulatory effect of these hormones on the expression of nsHbs (Hunt et al., 2001; Ross et al., 2004; Bustos-Sanmamed et al., 2011). Cytokinin-triggered changes in the expression of certain nsHbs have been described for several plant models (Ross et al., 2004; Bustos-Sanmamed et al., 2011). Moreover, transgenic and mutant plants with altered levels of particular nsHb classes have frequently displayed alterations in plant responses typically controlled by cytokinins (Hunt et al., 2001; Wang et al., 2011). For instance, marked changes in shoot organogenesis and altered expression of genes associated with cytokinin perception and signaling have been observed in *Arabidopsis* lines silencing or overexpressing class 1 or class 2 nsHbs (Wang et al., 2011). In the transgenic lines overexpressing nsHbs, cytokinin feedback repressors (Type-A ARRs) were repressed, whereas cytokinin activators (Type-B ARRs) and receptors were stimulated (**Figure 3**), culminating in a higher sensitivity of the tissues to the cytokinin-induced shoot organogenesis (Wang et al., 2011). Unfortunately, NO content was not measured in these transgenic lines; therefore, a direct correlation between

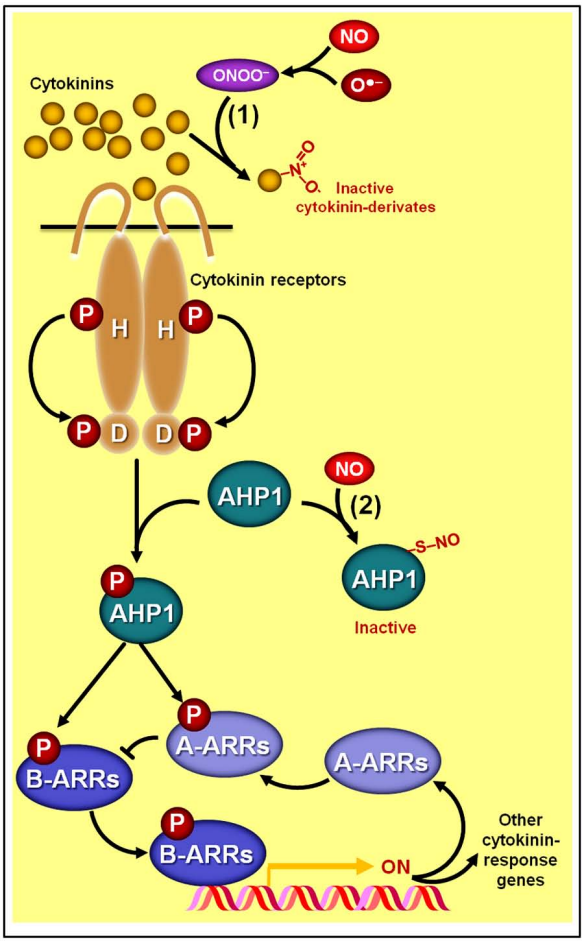

FIGURE 5 | Schematic representation of NO–cytokinin antagonistic interactions. (1) Certain cytokinin species such as zeatin may chemically react with peroxynitrite ($ONOO^-$), producing derivates with virtually no biological activity. (2) NO might also negatively impact cytokinin signaling since the protein HISTIDINE PHOSPHOTRANSFER PROTEIN 1 (AHP1), a key element in the phosphorelay mechanism involved in cytokinin transduction in *Arabidopsis*, may undergo *S*-nitrosylation at cys-115, rendering this protein incapable of transferring phosphoryl groups from the cytokinin receptors to the ARABIDOPSIS RESPONSE REGULATORs (ARRs). Protein *S*-nitrosylation and phosphorylation are represented by "–S–NO" and "P," respectively.

the higher responsiveness to cytokinins observed in nsHb overexpressing lines and their possibly lower NO levels could not be established.

NO AND ABSCISIC ACID INTERACTIONS

Both important "stress-related" molecules, NO and ABA intensively crosstalk during certain signaling cascades triggered by environmental challenges, such as water limitation and UV-B radiation, which ultimately leads to the induction of plant adaptive responses, such as stomatal closure and antioxidant defenses (Neill et al., 2008a; Tossi et al., 2009; Hancock et al., 2011). During the induction of these plant stress responses, NO mainly acts as a downstream element in the ABA signaling pathway since the impairment in NO production or its removal from tissues usually decreases or even eliminates ABA responses while the inhibition of ABA production typically does not affect the induction of these responses by exogenous NO application. On the other hand, during the regulation of certain developmental events not directly linked to plant stress responses, such as seed dormancy breaking, NO seems to counteract ABA effects (Bethke et al., 2006; Lozano-Juste and Leon, 2010a,b), suggesting a certain level of specificity in the NO–ABA interaction mechanisms, which may depend on the physiological events under analysis (e.g., stomatal closure *versus* seed dormancy release) or even the type of plant cell, tissue, or organ considered (e.g., guard cell *versus* seed tissues).

In some cases, such as during the induction of stomatal closure (Neill et al., 2002; Desikan et al., 2004; Bright et al., 2006) and during the up-regulation of the gene transcription and activities of antioxidant enzymes (Zhang et al., 2007; Lu et al., 2009; Zhang et al., 2009), ABA-induced NO generation seems to depend on H_2O_2 synthesis, suggesting this ROS as a mediator in NO-dependent ABA responses (**Figure 6**). In addition, the calcium/calmodulin system and MAPKs have also being identified as downstream elements of NO signaling during the regulation of plant antioxidant defenses induced either by ABA or H_2O_2 (Zhang et al., 2007; Sang et al., 2008). Moreover, cGMP has also been demonstrated to participate in NO-dependent ABA signaling, apparently acting downstream of NO and upstream of cytosolic Ca^{2+} (**Figure 6**; Dubovskaya et al., 2011). Similarly, type 2C protein phosphatases (PP2Cs), which acts as negative regulators of ABA signaling, have also been suggested to play a role as putative crosstalk elements between ABA receptors and NO-mediated ABA signal transduction, possibly acting downstream of NO in the complex networks controlling ABA-triggered stomatal closure (Desikan et al., 2002).

Since the discovery that NO scavengers could reduce ABA-induced stomata closure in turgid leaves of different plant species (Garcia-Mata and Lamattina, 2002; Neill et al., 2002), intensive research has been dedicated to characterize the mechanisms underlying the interplay between these two molecules in guard cell signaling networks (reviewed in Neill et al., 2008a; Hancock et al., 2011; Simontacchi et al., 2013), leading to the identification of several NO targets during the ABA-induced guard cell responses. Among these targets, plasma membrane calcium-dependent anion channels and inward-rectifying K^+ channels have been demonstrated to be activated and deactivated, respectively, by NO as a consequence of increases in guard cell cytoplasmatic Ca^{2+} levels (**Figure 6**) due to NO-triggered release of this anion from intercellular stores (Garcia-Mata et al., 2003).

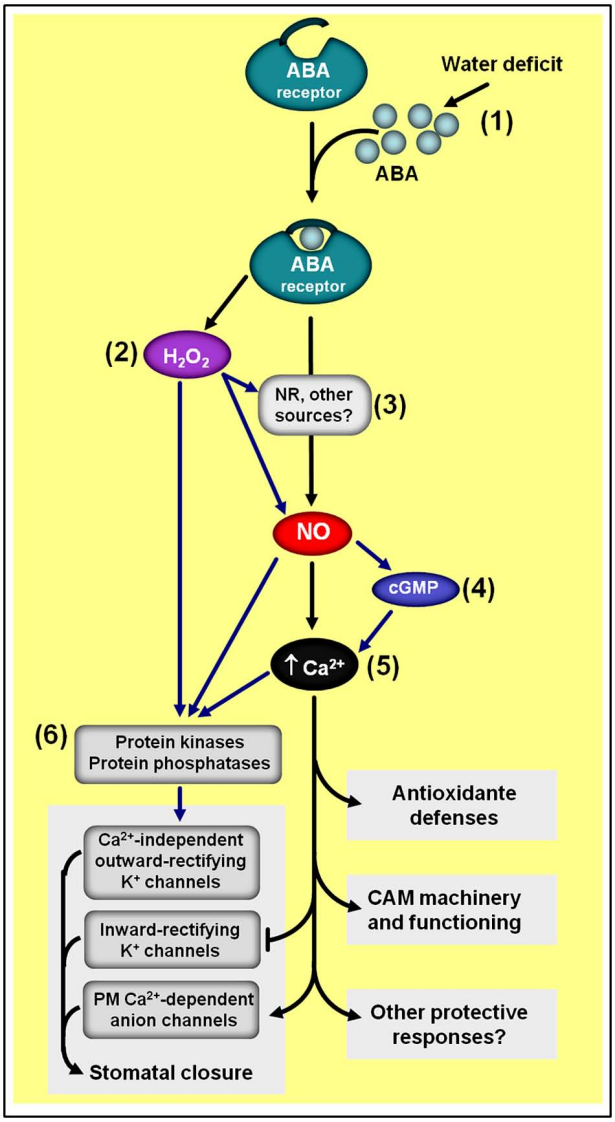

FIGURE 6 | Simplified schematic representation of NO–ABA interactions during defense responses to water shortage. (1) Water deficiency usually increases endogenous ABA levels. (2) ABA-induced NO generation depends on hydrogen peroxide (H_2O_2) synthesis. (3) NR seems to be one of the main sources of ABA-induced NO production. (4) NO-triggered changes in cytosolic calcium (Ca^{2+}) seem to involve cyclic guanosine monophosphate (cGMP). (5) The calcium/calmodulin system is a key downstream element of NO/ABA signaling. (6) Protein kinases and phosphatases are typical targets of H_2O_2, NO, and Ca^{2+}/calmodulin during ABA-induced responses. Black arrows indicate signaling steps shared by all three drought responses considered in the scheme (i.e., stomatal closure, antioxidant defenses, and Crassulacean acid metabolism induction). Blue arrows indicate some steps currently described only for the regulation of stomatal closure and/or antioxidant defenses. ABA- and NO-independent signaling pathways are not represented in this schematic representation.

Evidence for the involvement of protein phosphorylation upstream of intracellular calcium release has also been obtained, implicating protein kinases as additional targets of NO action

within ABA-regulated guard cell signaling (Sokolovski et al., 2005). Moreover, NO has also been reported to directly modulate calcium-independent outward-rectifying K^+ channels possibly by post-translationally modifying these channels or closely associated regulatory proteins (Sokolovski and Blatt, 2004). As a final consequence, this NO-dependent modulation of both Ca^{2+}-dependent and Ca^{2+}-independent ion channels at the plasma membrane of guard cells facilitates osmotic solute loss, thereby reducing guard cell turgor and promoting stomatal closure.

It is worth mentioning that NO has been suggested to play a role as a second messenger shared by multiple hormonal signaling cascades involved in the intricate guard cell network responsible for coordinating stomatal movement in higher plants, mediating not only the ABA signal but also ethylene (Liu et al., 2010), salicylic acid (SA; Hao et al., 2010), methyl jasmonate (Saito et al., 2009), auxin, and cytokinins (Xiao-Ping and Xi-Gui, 2006). Curiously, though, NO apparently is not an absolute requirement during the ABA signaling cascades leading to stomatal closure (Ribeiro et al., 2009) or the inhibition of light-induced stomatal opening (Yan et al., 2007; Yang et al., 2008); therefore, the existence of both NO-dependent and NO-independent pathways in ABA-induced guard cell responses is currently being suggested. Of course, more studies are clearly needed to better characterize a possible integrative, but apparently non-essential, role of NO during the regulation of stomatal movements by distinct environmental and hormonal stimuli.

At least in bromeliads, NO and ABA also seem to intensively interact to control Crassulacean acid metabolism (CAM) expression (Freschi et al., 2010; Mioto and Mercier, 2013), which, in turn, facilitates the survival of these plants under water- and nutrient-limited environments. As during the regulation of stomatal movements, NO apparently acts downstream of ABA and upstream of cytosolic calcium in the ABA-dependent signaling cascade leading to the up-regulation of the CAM machinery (**Figure 6**), and does not participate in the ABA-independent pathway also responsible for the regulation of this plant stress response (Freschi et al., 2010). The regulation of CAM expression in bromeliads as well as the control of stomata movements in *Arabidopsis* seem to have NR activity as the main source of the ABA-induced NO production (Desikan et al., 2002; Freschi et al., 2010).

While a number of pharmacological and genetic studies have reported higher endogenous NO levels following increases in plant tissue ABA concentration (i.e., NO action downstream of ABA; Zhang et al., 2009), NO-triggered changes in ABA biosynthesis and catabolism (i.e., NO action upstream of ABA) have rarely been described. In one of the few examples, Liu et al. (2009) reported that during the seed dormancy breaking in *Arabidopsis*, a rapid accumulation of NO in the endosperm layer preceded a decrease in ABA concentration, which was associated with a pronounced rise in the transcript and protein levels of the ABA 8′-hydroxylase CYP707A2, a key enzyme in ABA catabolism. Moreover, exogenous NO and the NO scavenger carboxy-PTIO (cPTIO), respectively, induced and impaired *CYP707A2* transcript accumulation during the imbibition period (Liu et al., 2009), further suggesting that the promotive effect of NO on seed dormancy break might indeed be associated with a stimulation of ABA catabolism.

In addition to modulating ABA catabolism, NO has also been described to affect the sensitivity of plant cells to ABA (Bethke et al., 2006; Lozano-Juste and Leon, 2010a,b). Bethke et al. (2006) reported that the NO donor SNP enhanced germination of dormant *Arabidopsis* seeds by decreasing the seed sensitivity to exogenous ABA. More recently, genetic evidence supporting this inhibitory effect of NO on ABA sensitivity was obtained by Lozano-Juste and Leon (2010a,b), who observed that the depletion of endogenous NO levels resulting from the generation of the *nia1,2noa1-2 Arabidopsis* triple mutant clearly led to ABA hypersensitivity. Among other features, this triple mutant displayed enhanced seed dormancy, decreased seed germination, and reduced seedling establishment in the presence of exogenous ABA, reinforcing the hypothesis that NO production during seed germination and initial seedling development counteracts the ABA inhibitory effects on these events. Interestingly, this ABA hypersensitivity continued through the post-germinative vegetative development of this triple mutant, as evidenced by the presence of increased expression of ABA-responsive genes, extreme drought resistance phenotype as well as higher responsiveness to ABA during stomatal closure (Lozano-Juste and Leon, 2010a,b). Curiously, dehydration- and ABA-dependent stomatal closure normally occurred in the presence of undetectable NO production in guard cells, corroborating the existence of a NO-independent pathway in this guard cell response (Ribeiro et al., 2009). Whether NO exerts its effects directly on ABA receptors or on some downstream element of ABA signaling cascade is obviously an important question that remains to be answered.

NO AND GIBBERELLINS INTERACTIONS

Nitric oxide has also been reported to influence several plant developmental events in which GAs play crucial roles, such as seed germination, hypocotyl elongation, acquisition of photomorphogenic traits, primary root growth, reorientation, and growth of pollen tubes, among others (Beligni and Lamattina, 2000; Prado et al., 2008; Tonón et al., 2010; Leon and Lozano-Juste, 2011); however, thus far, the actual interaction between NO and GAs has been described for only a limited number of these physiological events. In fact, most of our current knowledge of the mechanisms underlying the interplay between GAs and NO is restricted to the regulation of seed germination (Beligni et al., 2002; Bethke et al., 2007) and the inhibition of hypocotyl elongation during seedling de-etiolation (Leon and Lozano-Juste, 2011). During the control of these responses, NO has been described to act upstream of GA (Bethke et al., 2007), regulating both GA biosynthesis and perception/transduction (Leon and Lozano-Juste, 2011).

A certain level of antagonism between NO and GAs has been observed for most of the physiological processes in which both of these signaling compounds participate. A mounting body of evidence has indicated that DELLA proteins apparently represent a key crosstalk component between GA and NO signaling interactions (**Figure 7**; Leon and Lozano-Juste, 2011). DELLA proteins are a relatively small family of transcriptional regulators notably important for the integration of diverse hormonal signals, such

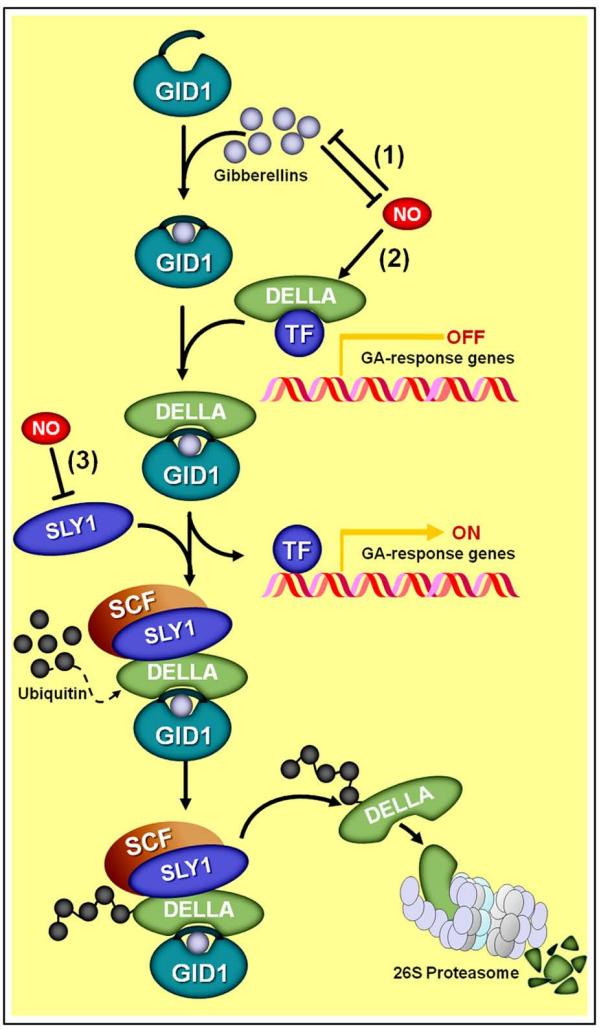

FIGURE 7 | Schematic representation of NO–gibberellin antagonistic interactions. (1) A mutual antagonism controls the endogenous levels of NO and gibberellins in *Arabidopsis* seedlings. (2) Additionally, NO negatively influences GA signaling by promoting the accumulation of DELLA proteins, whose presence represses the transcription of GA-regulated genes. Since the degradation of DELLAs through SCF-26S proteasome-mediated proteolysis depends on the interaction of these proteins with the complex formed by active gibberellin molecules associated with the receptor GA INSENSITIVE DWARF1 (GID1) and the E3 ubiquitin ligase SLEEPY1 (SLY1), the NO-driven increase in DELLAs and reduction in SLY1 abundance (3) negatively impacts the transduction of the GA signal.

as GAs, ethylene, jasmonate (JA), and ABA (Achard et al., 2003; Gao et al., 2011; Ross et al., 2011). During GA signaling transduction, for instance, the hormonal molecules interact with GA INSENSITIVE DWARF1 (GID1) receptors, which, in turn, binds a DELLA protein and subsequently directs the GA–GID1–DELLA complex to the E3 ubiquitin ligase SLEEPY1 (SLY1), thereby promoting DELLA degradation at the proteasome (**Figure 7**). Given that DELLAs mainly act by repressing the transcription of GA-regulated genes, the perception and transduction of the GA signal leads, as a final result, to a decrease in DELLA concentration into the cell and a consequent induction of GA-responsive genes.

Interestingly, recent studies have indicated that NO triggers the opposite effect on cellular DELLA concentration, promoting the accumulation of this protein and a consequent negative impact on GA signal transduction (**Figure 7**). Essentially, this NO-driven DELLA accumulation can be interpreted as a reduction in tissue sensitivity to GA since a larger number of GA–GID1–DELLA complexes will need to be formed in order to mark an adequate quantity of DELLA proteins for proteasome degradation, thereby leading to a satisfactory level of transcriptional de-repression of GA-regulated genes. This differential effect of NO and GAs on DELLA regulation might account, at least in part, for the antagonism observed between these two signaling compounds during the regulation of physiological processes, such as hypocotyl elongation (Leon and Lozano-Juste, 2011) and primary root growth (Fernández-Marcos et al., 2012) in *Arabidopsis*.

In addition, studies performed on *nia1,2noa1-2* seedlings revealed that this NO-deficient mutant presents defective DELLA accumulation associated with an up-regulation of the E3 ubiquitin ligase SLY1 (**Figure 7**), resulting in increased GA sensitivity and deficient de-etiolation under red light (Leon and Lozano-Juste, 2011). Further emphasizing the potential role for DELLAs in the GA–NO antagonistic interactions, exogenous NO was also demonstrated to induce the accumulation of GA-regulated DELLA proteins (Leon and Lozano-Juste, 2011), very likely by negatively regulating the GID1–SLY1 system of DELLA tagging for degradation (**Figure 7**). However, as pointed out by Leon and Lozano-Juste (2011), the regulation of DELLA turnover and activity may represent the main but not the only target for NO action in regulating plant growth and other GA-mediated developmental responses since DELLA-independent mechanisms might also be implicated.

Besides the negative action of NO on GA signaling network, a mutual antagonism controlling the endogenous levels of these two signaling molecules has also recently been proposed (**Figure 7**) (Leon and Lozano-Juste, 2011). Supporting this suggestion, etiolated seedlings of the GA-deficient *Arabidopsis* mutant *ga1-3* have been shown to exhibit NO levels significantly higher than those observed in the WT genotype. Moreover, both *ga1-3* mutant and WT seedlings showed reduced NO levels after GA_3 supplementation, thereby suggesting that GAs negatively modulates NO production (Leon and Lozano-Juste, 2011). On the other hand, WT *Arabidopsis* seedlings treated with SNP presented a significant reduction in endogenous GA levels (Leon and Lozano-Juste, 2011). Based on a detailed analysis of the expression of *Arabidopsis* genes involved in GA biosynthesis (*GA20oxidase* and *GA3oxidase*) and catabolism (*GA2oxidase*), *GA20ox3* was identified as the only gene significantly up-regulated in the NO-deficient *nia1,2noa1-2* mutant and down-regulated in NO-treated WT seedlings (Leon and Lozano-Juste, 2011).

Under certain circumstances, however, NO seems to play a stimulatory rather than inhibitory role in the GA biosynthetic machinery (Bethke et al., 2007). Exemplifying such a synergist relationship, Bethke et al. (2007) reported that NO generation was required for the transcription of two *GA3oxidase* genes (*GA3ox1* and *GA3ox2*) during the *Arabidopsis* seed dormancy breaking. Another indication of the positive interaction between GA and NO has recently been reported in wheat roots, for which the

SNP-induced apical growth was associated with increased GA_3 levels (He et al., 2012).

Apart from the above-mentioned evidence of NO acting upstream of GA, a certain level of uncertainty remains as to whether NO and GA actually share a common signaling route or just act through parallel, independent cascades during the regulation of some plant responses. During seed dormancy breaking, for instance, although there is virtually no doubt that both of these signal molecules promote germination in a number of species (Giba et al., 1998; Beligni and Lamattina, 2000; Kopyra and Gwozdz, 2003), whether and how NO and GA interact during this process still needs further characterization.

In fact, whereas a mounting body of evidence indicates that NO selectively interferes in some specific GA-induced events associated with the seed germination process, such as the longevity of cereal aleurone cells (Beligni et al., 2002), transcription of Myb transcription factor (GAMYB), and amylase synthesis (Wu et al., 2013), for some other responses associated with the germination process, no indications of additive or antagonistic responses have been found when both GA and NO were exogenously applied (Zhang et al., 2005). In addition, a rapid burst in NO production has been detected during early seed germination (Simontacchi et al., 2004), which has been speculated to be temporally dissociated from the action of GAs at later stages of seed germination (Zhang et al., 2005).

Regardless of whether or not NO and GA share a common signaling cascade during seed dormancy breaking, the stimulation of seed germination by either of these substances can be blocked by sufficiently high concentrations of ABA (Bethke et al., 2004, 2006; Sarath et al., 2006; Dong et al., 2012). Considering that NO may stimulate germination not only by breaking seed dormancy but also by alleviating the influence of environmental factors inhibitory to the germination process (Bethke et al., 2007), a NO-hormonal network much more complex than the interaction between NO, GA, and ABA might possibly be involved in the regulation of this critically important step in the plant life cycle.

NO AND ETHYLENE INTERACTIONS

A significant number of the currently available reports on the interaction between NO and ethylene suggest an antagonistic relationship between these two gaseous molecules (Leshem et al., 1998; Lamattina et al., 2003; Manjunatha et al., 2010). The first and presently most explored plant phenomenon in which NO was demonstrated to counteract ethylene production and action is the control of fruit ripening and the regulation of leaf and flower senescence (Leshem et al., 1998; Manjunatha et al., 2010). For these responses, ethylene has long been identified as a key promotive signal, and a large number of reports indicate that the production and perception mechanisms of this plant hormone are under strict regulation, depending not only on the plant developmental program but also on a number of environmental factors (Grbić and Bleecker, 1995; Fischer, 2012). Additional studies revealed that exogenous application of NO, either by direct fumigation or by means of NO-releasing chemicals, delays senescence of both vegetative and reproductive organs by negatively regulating a number of elements involved in ethylene production (Leshem and Haramaty, 1996; Leshem et al., 1998; Wills et al., 2000; Zhu et al., 2006; Liu et al., 2007; Manjunatha et al., 2010, 2012). Corroborating this pharmacological evidence, measurements of ethylene and NO emission during either fruit ripening (Leshem et al., 1998; Leshem and Pinchasov, 2000) or plant senescence (Magalhães et al., 2000; Corpas et al., 2004) revealed an opposite trend between these gases, in which ethylene production increases, whereas NO levels decrease during the induction and establishment of these processes.

Recent studies have revealed that the inhibition of fruit ethylene production by NO may be attributed to a reduction in the transcript level and/or activity of key ethylene biosynthetic enzymes (Manjunatha et al., 2010). In vegetative and reproductive plant tissues, ethylene production depends on the conversion of the *S*-adenosyl methionine (SAM), derived from "Yang cycle," into the immediate ethylene precursor 1-aminocyclopropane 1-carboxylic acid (ACC) through ACC synthase (ACS) activity (**Figure 8**). The ACC formed may be subsequently converted to ethylene due to the activity of a second enzyme, the ACC oxidase (ACO; Yang and Hoffman, 1984). Since the abundance of ACC, ACS, and ACO in plant tissues represents a critical aspect for determining ethylene production rates (Barry et al., 1996; Barry et al., 2000), an inhibitory effect of NO on any of these elements can be expected to be an efficient mechanism for down-regulating ethylene synthesis.

As revealed by a series of studies on climacteric fruits, exogenous NO indeed has the capacity to modulate both the transcription and the activity of both ACS and ACO (**Figure 8**), consequently impacting not only the levels of ethylene production but also the accumulation of ACC (Manjunatha et al., 2010). In tomato fruits, for instance, although the expression of all ACS homologs remained virtually unchanged following NO fumigation, the transcript abundance of ACO genes, such as *LeACO1*, *LeACOH2*, and *LeACO4*, and the levels of ethylene emission were reduced and/or delayed when NO was applied before the start of the ripening process (Eum et al., 2009). In banana fruits, on the other hand, NO negatively impacted the expression of both ACS and ACO homologs, leading to a reduction in ACO activity and ethylene emission as well as an accumulation of ACC (Cheng et al., 2009).

Apart from controlling the transcript levels of ACS and ACO, NO may also regulate ACS activity via *S*-nitrosylation (Abat and Deswal, 2009) and influence ACO activity by a mechanism involving the direct binding of NO to the enzyme, resulting in the ACO–NO binary complex, which is then chelated by ACC to produce the ternary stable complex ACO–NO–ACC (**Figure 8**) (Tierney et al., 2005; Zhu et al., 2006; Manjunatha et al., 2010). Currently, the impacts of *S*-nitrosylation on ACS activity remain uncharacterized, and the occurrence of the ACO–NO–ACC ternary complex is exclusively described during *in vitro* studies conducted on recombinant ACO (Tierney et al., 2005); therefore, the actual *in vivo* implications of such regulatory mechanisms still need further elucidation. Nevertheless, the hypothetical formation of an ACO–NO–ACC complex has already been inferred as possibly responsible for the reduction of ACO activity in climacteric peach (*Prunus persica*) fruits subjected to NO fumigation, which resulted in a concomitant decrease in ethylene emission and accumulation of ACC (Zhu et al., 2006). In this specific case, the NO-induced reduction of ACO activity was accompanied by an increment in

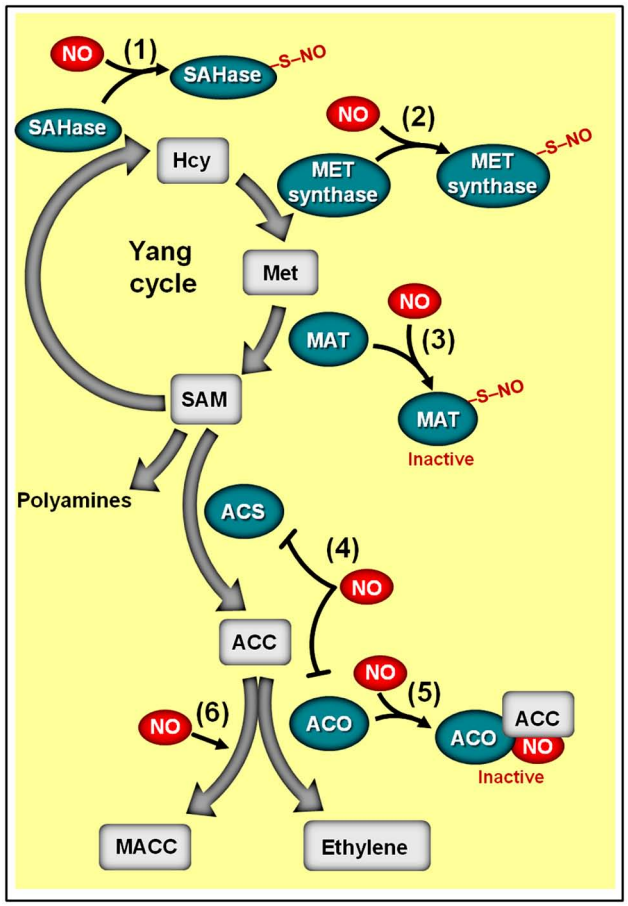

FIGURE 8 | Schematic representation of NO-ethylene antagonistic interactions. The methylmethionine cycle enzymes adenosyl homocysteinase (SAHase) (1) and methionine synthase (MET synthase) (2), whose activities are responsible for the production of homocysteinase (Hcy) and methionine (Met), respectively, may undergo *S*-nitrosylation. (3) Additionally, the activity of the *Arabidopsis* methionine adenosyltransferase 1 (MAT1) can be suppressed by *S*-nitrosylation, thereby repressing the conversion of methionine (Met) to *S*-adenosyl methionine (SAM). (4) In ripening climacteric fruits, NO has been shown to inhibit the transcript levels of 1-aminocyclopropane 1-carboxylic acid (ACC) synthase (ACS) and/or ACC oxidase (ACO). (5) NO can also inhibit ACO activity by directly binding this enzyme, resulting in the ACO–NO binary complex, which subsequently originates a ternary stable complex ACO–NO–ACC. (6) NO-driven accumulation of non-volatile ACC metabolite 1-malonyl aminocyclopropane-1-carboxylic acid (MACC) has also been reported. Biosynthetic enzymes are represented with green ovals and metabolic substrates and products with gray rectangles. Protein *S*-nitrosylation is represented by "–S–NO." Note that the impact of *S*-nitrosylation on the activities of SAHase, MET synthase, and ACS remains to be determined.

the accumulation of the non-volatile ACC metabolite 1-malonyl aminocyclopropane-1-carboxylic acid (MACC; **Figure 8**), which was interpreted as a secondary effect of NO during the ripening of these fruits (Zhu et al., 2006).

Besides stimulating the irreversible conversion of ACC into MACC, NO may also negatively impact the turnover of SAM, which is the main precursor molecule for ACC synthesis. Supporting this assumption, proteomic analysis of *Arabidopsis* plants revealed that the methylmethionine cycle enzymes adenosyl homocysteinase (SAHase), methionine synthase (MET synthase) and methionine adenosyltransferase (MAT, also known as SAM synthase), whose activities are responsible for the production of homocysteinase (HCY), methionine (Met), and SAM, respectively, may undergo *S*-nitrosylation (**Figure 8**). In addition, similar analyses conducted on GSNO-treated protein extracts of *Kalanchoe pinnata* (Abat et al., 2008) and *Brassica juncea* (Abat and Deswal, 2009) also identified cobalamin-independent MET synthases as a common target of *S*-nitrosylation. Whereas the influence of *S*-nitrosylation on the activities of SAHase and MET synthase has yet to be determined, a detailed study conducted by Lindermayr et al. (2006) revealed that the activity of MAT1, one of the three *Arabidopsis* MAT isoforms, is indeed suppressed via *S*-nitrosylation at cys-114, having as a logical consequence the depletion of the SAM pool and a reduction in ethylene production. Curiously, the study conducted by Lindermayr et al. (2006) was the first detailed characterization of *S*-nitrosylation in plant systems, opening up a new window of opportunities for accessing the actual relevance of this NO-dependent post-translational regulatory mechanism in plant signaling.

In contrast to the above-mentioned evidence of an antagonistic relationship between NO and ethylene during the maturation, senescence, and abscission of plant organs, a number of reports have also indicated that NO donors, such as SNP, might sometimes stimulate, rather than negate, ethylene production in certain plant materials, such as non-senescent leaf tissues of *Arabidopsis*, tobacco, and maize (Magalhães et al., 2000; Ederli et al., 2006; Wang et al., 2006; Mur et al., 2008; Ahlfors et al., 2009) and apple embryos (Gniazdowska et al., 2007). In tobacco leaves, for instance, SNP infiltration has been show to stimulate ACS expression (Ederli et al., 2006; Mur et al., 2008), whereas in *Arabidopsis* roots the application of GSNO positively impacted the transcript levels of not only ACS but also other key ethylene biosynthetic enzymes, such as SAM synthetases, ACOs, and 5-methylthioribose kinase (MTK; Garcia et al., 2011). Further emphasizing a stimulatory influence of NO on ethylene biosynthesis, ethylene production is usually elevated when the NO accumulation is promoted via suppression of nsHbs gene expression (Manac'h-Little et al., 2005; Hebelstrup et al., 2012). Similarly, the increased NO production observed in transgenic tobacco lines expressing mammalian NOS were accompanied by a higher expression of ACO and some other ethylene-related genes (Chun et al., 2012). Moreover, a concomitant increase in both ethylene and NO emission has been consistently observed both in tobacco leaves undergoing bacterially triggered hypersensitive response (Mur et al., 2012b) and in *Arabidopsis* and cucumber (*Cucumis sativus*) roots subjected to Fe deficiency (Garcia et al., 2011).

Besides these indications of a positive influence of NO on ethylene production, some data also seem to support a stimulatory role of ethylene on NO production under certain circumstances (Garcia et al., 2011). Earlier in the research of NO–ethylene interaction in plants, Leshem and Haramaty (1996) reported that exogenous ACC induced significant increases in both ethylene and NO emission in pea (*Pisum sativum*) leaves. More recently, Garcia et al. (2011) have also detected increased NO levels in the root subapical region of *Arabidopsis* and cucumber plants exposed to

ACC. In addition, these authors reported that inhibitors of ethylene biosynthesis and action completely abolished the increases in NO levels in roots of plants subjected to Fe deficiency. In contrast, ethylene supplementation or depletion, respectively, repressed and promoted NO production during the abscission of mature olive fruits (Parra-Lobato and Gomez-Jimenez, 2011), which apparently indicates that under certain circumstances ethylene may negatively, rather than positively, impact the endogenous NO levels. In agreement with this, ethylene has sometimes been shown to induce class 1 nsHbs (Qu et al., 2006; Bustos-Sanmamed et al., 2011), which in turn may lead to reductions in tissue concentration of NO.

Surprisingly, the possible influence of NO on ethylene signal transduction elements has remained virtually unexplored, both during antagonistic (e.g., fruit ripening and leaf senescence) and synergistic (e.g., plant defense to biotic stresses and Fe deficiency) interactions between these signaling substances. Therefore, it is currently unknown whether NO might regulate the transcripts levels or activities of receptors, signal transduction proteins and/or transcription factors involved in ethylene signaling, which would very likely impact the sensitivity of the plant tissues to this plant hormone. In one of the few studies on this line, Niu and Guo (2012) demonstrated that the dark-induced early senescence phenotype of the *Arabidopsis* NO-deficient mutant *noa1* was suppressed by mutation in ETHYLENE INSENSITIVE 2 (EIN2) and indicated that this protein might act downstream of NO signaling, possibly playing a key role as a crosstalk point between ethylene and NO signaling cascades.

INTERACTIONS BETWEEN NO AND OTHER PLANT HORMONES

Besides interacting with the five "classical" phytohormone classes, NO has also been reported to crosstalk with other plant hormones, including JAs, SA, polyamines, and brassinosteroids. Some of these interactions, such as the interplay between NO, SA, and JA in plant defense responses, have been investigated in great detail, uncovering impressively complex NO–phytohormone interaction networks. A detailed discussion about these interactions is beyond the scope of the present work; instead, just some brief, general comments, and examples of these NO–phytohormone crosstalks will be provided below.

As recently reviewed by Yu et al. (2012) and Mur et al. (2013), during the induction of plant defense responses against biotic challenges, NO positively impacts the production of both SA and JA (Feechan et al., 2005; Chun et al., 2012; Mur et al., 2012b) and, at the same time, NO modulates SA signaling by controlling the oligomerization status of the translational activator NON-EXPRESSER OF PATHOGENESIS-RELATED GENE1 (NPR1) via *S*-nitrosylation at cys156 (Tada et al., 2008; **Figure 9**). *S*-nitrosylation of NPR1 facilitates its oligomerization (Tada et al., 2008) and permanence in the cytosol (Fu et al., 2012), where it may interact with SA receptors (NPR3/4). Following such interaction with NPR3/4, the *S*-nitrosylated cys156 of NPR1 is reduced (Tada et al., 2008), promoting NPR1 monomer formation and its consequent migration to the nucleus, where this protein may interact with several TGA-class transcription factors that subsequently activate promoters of SA-responsive genes (Mur et al., 2012b; **Figure 9**). In contrast, the presence of the *S*-nitrosylated, oligomeric form of NPR1 in the cytosol facilitates the repression of JA-triggered responses (Spoel et al., 2003). Consequently, this NO-dependent PTM of NPR1 seems to play a key integrative role during the hormonal signaling cascades leading to coordinated plant immunity responses (Yu et al., 2012; Mur et al., 2013). In parallel, *S*-nitrosylation of SA-BINDING PROTEIN 3 (SABP3) at cys280, which takes place during late stages of bacterial infection, represses its capacity to bind SA, and antagonizes the expression of plant immunity responses (Wang et al., 2009), thereby representing a negative feedback loop apparently essential for the correct regulation of SA-modulated plant defense against biotic challenges (**Figure 9**).

Accumulating evidence indicates that NO might also mediate both developmental and stress responses induced by polyamines (Wimalasekera et al., 2011). Briefly, very rapid NO production has been observed in plant tissues exposed to mM concentration of polyamines (Tun et al., 2006), which has sometimes been interpreted as an indication of a potential NO biosynthetic pathway involving the catabolism of these plant hormones (Wimalasekera et al., 2011). Given the absence of a lag phase between the application of polyamines and the rise in NO endogenous levels (Tun et al., 2006), it is currently assumed that these hormones might be directly converted to NO by the action of one or more enzymes, whose identities are yet to be determined (Wimalasekera et al., 2011). So far, it is only known that the polyamine-induced NO production can be quenched by mammalian NOS inhibitors and is not affected in *Arabidopsis* NR-deficient mutants (Tun et al., 2006; Wimalasekera et al., 2011). Whether polyamines act as substrates, cofactors, or signals for promoting NO synthesis also needs to be better determined; therefore, monitoring the formation of ^{15}NO from isotopic-labeled polyamines in plant tissues or extracts seems an important experiment in future studies. A possible influence of NO on polyamine metabolism has been demonstrated in some studies (Fan et al., 2013) but not in others (Arasimowicz-Jelonek et al., 2009); consequently, this topic still deserves further investigation.

Considering that polyamines and ethylene share SAM as a common precursor, all the basic NO-dependent mechanisms controlling the SAM pools discussed earlier in this review (**Figure 8**) might also indirectly affect polyamine synthesis in plants. In addition, we also need to keep in mind that L-arginine is substrate for the production of polyamines, via arginase and arginine decarboxylase activities, as well as NO, via NOS-like activities; therefore, the availability of this particular amino acid might also influence NO/polyamine connections in plants and other organisms. In mammals, for instance, the occurrence of an arginine switch, in which NOS and arginase compete for arginine, seems to be supported by a great deal of experimental evidence (Satriano, 2004). In parallel, literature data in the animal field also indicates that polyamines such as spermidine and spermine influence NO production via NOS activity during diverse physiological responses (Guerra et al., 2006), which may represent an important source of information to guide current and future research on the NO and polyamine interactions in plants. The polyamine precursor agmatine, for example, has been demonstrated to act either as an alternative substrate or a competitive

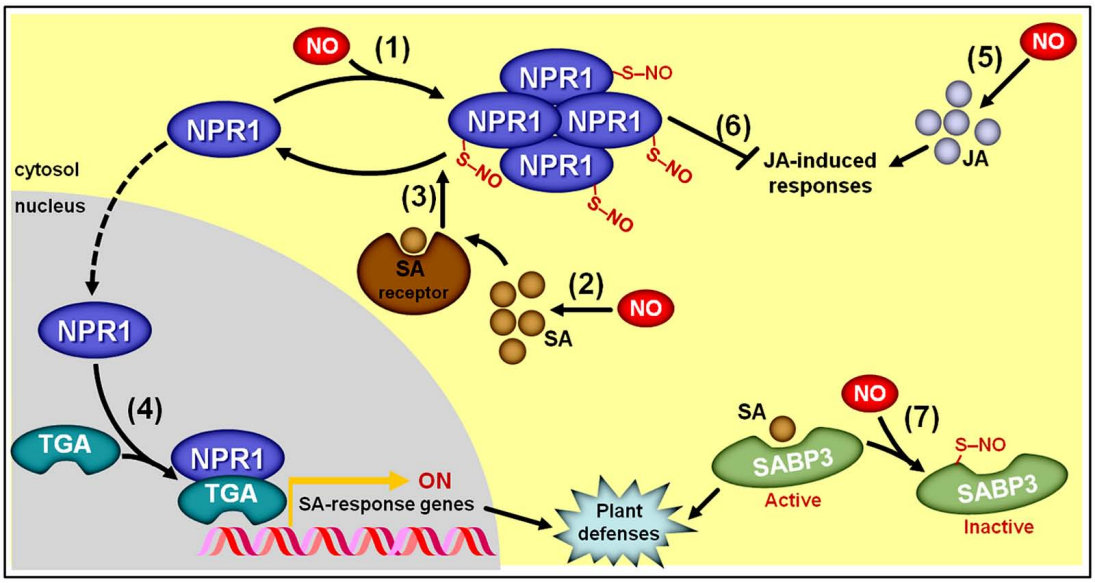

FIGURE 9 | Simplified schematic representation of NO, salicylic acid (SA), and jasmonic acid (JA) interactions during plant responses to biotic challenges. (1) *S*-nitrosylation of NON-EXPRESSER OF PATHOGENESIS-RELATED GENE1 (NPR1) at cys156 promotes its oligomerization and permanence in the cytosol. (2) NO stimulates SA biosynthesis. (3) Oligomeric NPR1 is denitrosylated following its interaction with SA receptors, which promotes the formation of monomeric NPR1. (4) Monomeric NPR1 translocates to the nucleus, where this protein binds TGA-class transcription factors, which subsequently activate promoters of SA-responsive genes. (5) NO also stimulates JA biosynthesis. (6) Cytosolic, oligomeric NPR1 represses JA-triggered responses. (7) At late stages of bacterial infection, *S*-nitrosylation of SA-binding protein 3 (SABP3) at cys280 represses its SA binding capacity, thereby promoting a negative feedback loop during the defense signaling pathway. Protein *S*-nitrosylation is represented by "–S–NO."

inhibitor of mammalian NOS, depending on the isoform or physiological process taken under consideration (Satriano, 2003; Raghavan and Dikshit, 2004), thereby indicating a possible role for this compound as an endogenous regulator of NO generation in mammals. In plants, the ameliorative effects of both polyamines and NO under stressful conditions (Arasimowicz-Jelonek et al., 2009; Wimalasekera et al., 2011; Gupta et al., 2013) might represent an important driving force to stimulate further studies on the interaction between these critically important signaling compounds.

Generation of information about whether and how NO and brassinosteroids interact has only recently begun (Zhang et al., 2011; Tossi et al., 2013). In one of these studies, Zhang et al. (2011) demonstrated that nM concentrations of brassinosteroids promoted rapid increases in the NO levels of leaf mesophyll cells, which together with some other evidence allowed the authors to place NO as a possible intermediate in the brassinosteroid-induced ABA biosynthesis in maize leaves. More recently, Tossi et al. (2013) reported that NR and NOS-like activities are probably involved in the brassinosteroid-induced NO production in *Arabidopsis* and that NO very likely mediates brassinosteroid-triggered modifications in plant root architecture.

SOME CONCLUSIONS AND MANY UNANSWERED QUESTIONS

Despite the methodological difficulties and conceptual complexity intrinsically involved in the elucidation of the exact mechanisms responsible for interconnecting plant hormones and NO signaling during the coordination of plant metabolism and development, some cutting edge insights into the NO–phytohormone crosstalks have recently been achieved.

Many downstream and upstream components of the NO signaling cascades have been identified, and NO-dependent PTMs, notably *S*-nitrosylation, have emerged as critical mechanisms controlling key elements involved in plant hormone production and signaling. As highlighted in the course of this review, by chemically modifying these hormone-related proteins, NO may modify plant hormone metabolism and signaling at multiple, diversified levels. The identification and functional analysis of the protein targets of NO-dependent PTMs and whose action determines the delicate hormonal homeostasis in plants has been, and will probably continue to be, an approach of upmost relevance in NO–phytohormone studies.

Currently, the physiological relevance of NO-dependent chemical modifications of phytohormone-related proteins has been poorly investigated *in planta*; therefore, this remains a rich area for future investigation. Clarifying how these NO-triggered PTMs, particularly *S*-nitrosylation and tyrosine nitration, actually control protein activity, subcellular localization as well as protein–protein, protein–DNA, protein–cofactors, or even protein–hormone binding capacity will inexorably involve the use of a wide range of experimental strategies and methodological approaches, some of which are currently available (e.g., overexpression of modified proteins in mutant genetic backgrounds) and others yet to be developed. Since some proteins are targets of multiple NO-dependent PTMs, sometimes even

involving different types of these chemical modifications (e.g., both *S*-nitrosylation and nitration; Lozano-Juste et al., 2011; Astier et al., 2012), it would be enlightening to determine the impacts of concomitant NO-triggered modifications on the same protein.

Moreover, characterizing how these target proteins are chemically modified by NO and NO-derivates at the right time and place seems to be another promising area of progress in NO–phytohormone interactions. Addressing this question inevitably implies dealing with several critical aspects of the NO physiology that still require further elucidation. Firstly, the basic mechanisms responsible for NO production, removal, and transport in plants continues to represent a critical impediment for advances in the clarification of how NO levels are temporally and spatially controlled by plant hormones and other stimuli. A fine-tuned equilibrium between NO production and removal (e.g., biosynthesis *versus* degradation, conjugation *versus* deconjugation) might possibly exist to determine both the localization and the concentration of NO and NO-derivates within the plant cells. Secondly, given the impressive diversity of target proteins, which are ubiquitously distributed within the plant cells, the existence of a certain subcellular compartmentation in NO production and action is an assumption that urgently needs to be investigated in greater detail. Moreover, a concentration-dependent action mode for NO has also been proposed (Mur et al., 2013), in which distinct responses may be triggered depending on the abundance of this free radical. Obviously, the development of more sensitive and specific means to determine the subcellular localization and concentration of NO and NO-derivates is critical for further advances in this area. Thirdly, considering that both *S*-nitrosylation and tyrosine nitration are apparently reversible events, more conclusive studies on the denitrosylation and denitration systems as well as the general turnover of *S*-nitrosylated and nitrated proteins in plant cells also seems a logical requirement for a deeper understanding of the dynamics of these regulatory processes. Similar to the action of protein phosphatases during the regulation of protein phosphorylation, denitrosylases and denitrases may possibly play an important role in defining the kinetics of the NO impacts on plant signaling cascades.

Another aspect that also deserves further attention is the potential existence of feed-forward cycles, in which NO modulates the production and/or signaling of specific plant hormones and these same hormonal species influence the machinery responsible for controlling NO endogenous levels. As described in the course of this review, accumulating pharmacological and genetic evidence demonstrates that representatives of virtually all classes of plant hormones may impact, at least at a certain degree, the endogenous concentration and/or distribution of NO and, also very frequently, literature data seems to indicate that changes in NO levels might trigger alterations on the metabolism and/or signaling of many, if not all, hormonal classes. It is not clear, however, whether these processes occur at the same place and time, which is critical for generating authentic feed-forward cycles involving these signaling substances. Naturally, a more complete characterization of the actual impacts of specific plant hormones on the NO biosynthetic and removal machinery (e.g., NR, NOA1, GSNOR, nsHbs) seems a key step in such research topic.

Additionally, as also discussed earlier in this review, NO might affect the signaling transduction of certain hormones, such as auxins and GAs, by modulating signaling elements (e.g., receptor, signaling transduction molecules) that impact the general dynamics of ubiquitination and proteasome-dependent degradation of repressor proteins (e.g., AUX/IAA and DELLA proteins). Interestingly, in animal systems, NO has consistently been shown to influence protein stability via regulation of ubiquitination and proteasome-dependent proteolysis (Hess et al., 2005) and, at least in humans, ubiquitin ligases themselves are targets of *S*-nitrosylation (Chung et al., 2004). Considering that several plant hormone signaling transduction mechanisms are based on the ubiquitination and subsequent proteasome-dependent degradation of repressor proteins, investigating whether NO might also directly affect this protein degradation labeling system in plants seems a promising venue for uncovering additional mechanisms possibly involved in NO signaling in plant systems.

Another intriguing question that remains to be answered is how plants can distinguish endogenously produced NO signals from the NO naturally present in the environment (e.g., atmosphere, rhizosphere). Whereas the gaseous and highly diffusible nature of NO may promote certain movement of this molecule inside the plant tissue and at the plant–environment interface, the high reactivity and inherent instability of NO may possibly limit the diffusion of this free radical through biological tissues. In several aspects, this seems a relevant and challenging question to be answered in the future.

Finally, we must remain open-minded to conceive increasingly complex NO–phytohormone interconnection nodes since new targets of NO-dependent PTMs and other upstream and downstream elements of NO signaling cascades will likely be identified in the future. At the same time, more complete pictures mechanistically explaining how multiple plant hormones may simultaneously interact with NO to control specific plant responses might also emerge, very likely leading to exciting new models of NO–phytohormone interaction networks. Moreover, this whole scenario will be further complicated when the intensive research conducted today in a restricted number of plant models (e.g., *Arabidopsis*, tomato, rice) is extended to a broader range of plant species and environmental contexts. Altogether, this knowledge will improve our ability to define the actual roles of NO during the regulation of the distinct plant responses controlled by this multipurpose signaling molecule and may also lead to new opportunities to manipulate NO–phytohormone interactions and, thus, regulate plant growth, development, and metabolism.

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Which role for nitric oxide in symbiotic N_2-fixing nodules: toxic by-product or useful signaling/metabolic intermediate?

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The interaction between legumes and rhizobia leads to the establishment of a symbiotic relationship characterized by the formation of new organs called nodules, in which bacteria have the ability to fix atmospheric nitrogen (N_2) via the nitrogenase activity. Significant nitric oxide (NO) production was evidenced in the N_2-fixing nodules suggesting that it may impact the symbiotic process. Indeed, NO was shown to be a potent inhibitor of nitrogenase activity and symbiotic N_2 fixation. It has also been shown that NO production is increased in hypoxic nodules and this production was supposed to be linked – via a nitrate/NO respiration process – with improved capacity of the nodules to maintain their energy status under hypoxic conditions. Other data suggest that NO might be a developmental signal involved in the induction of nodule senescence. Hence, the questions were raised of the toxic effects versus signaling/metabolic functions of NO, and of the regulation of NO levels compatible with nitrogenase activity. The present review analyses the different roles of NO in functioning nodules, and discusses the role of plant and bacterial (flavo)hemoglobins in the control of NO level in nodules.

Keywords: legume, nitric oxide, nitrogen fixation, rhizobium, symbiosis

INTRODUCTION

Nitric oxide (NO) is a gaseous molecule which was found to be involved in plant development, and response to biotic or abiotic stresses (Besson-Bard et al., 2008). NO production was also reported during symbiotic interactions, particularly in the nitrogen (N_2)-fixing symbiosis (NFS) between legumes and soil Gram-negative bacteria called rhizobia (Baudouin et al., 2006). The interaction between legumes and rhizobia leads to the establishment of a symbiotic relationship characterized by the formation of new differentiated organs called nodules, which provide a niche for bacterial N_2 fixation. In the nodules, bacteria released in plant cells differentiate into bacteroids with the ability to fix atmospheric N_2 via nitrogenase activity (Oldroyd and Downie, 2008). As nitrogenase is strongly inhibited by oxygen, N_2 fixation requires the microaerophilic conditions prevailing in the nodules (Appleby, 1992). Thus, nodule development occurs in changing oxygen conditions, shifting from a normoxic environment during symbiosis establishment to a microoxic one in functioning nodules. During the last decade, increasing evidence of the presence of NO during symbiosis, from early interaction steps between the plant and the bacterial partners to N_2-fixing and senescence steps in mature nodules, has been reported (for review, see Meilhoc et al., 2011). At later stages of the interaction, NO was observed to be produced in N_2-fixing nodules of *Medicago truncatula* and *M. sativa* particularly in bacteroid-containing cells (Baudouin et al., 2006; Pii et al., 2007). NO was also detected directly in mature nodules of *Lotus japonicus* (Shimoda et al., 2009), and indirectly through the detection of nitrosylleghemoglobin complexes in nodules of soybean and pea (Kanayama et al., 1990; Mathieu et al., 1998; Meakin et al., 2007). Interestingly, both the plant and the bacterial partners were shown to participate significantly in NO synthesis (Sanchez et al., 2010; Horchani et al., 2011).

The chemical nature, concentration, and location of NO might influence its biological role, and at high local concentration NO can become very toxic. NO was thus shown to inhibit the growth of *Sinorhizobium meliloti* in culture (Meilhoc et al., 2010), and the symbiotic N_2 fixation in legumes (Sasakura et al., 2006; Shimoda et al., 2009; Kato et al., 2010). However, more recently NO has been found to play a beneficial metabolic function for the maintenance of the energy status (Horchani et al., 2011), or to have a regulatory role in the regulation of N_2 metabolism (Melo et al., 2011) in functioning nodules. These observations raised the question of the role of NO in N_2-fixing nodules. This review focuses on the toxic versus metabolic roles of NO in symbiotic nodules, and discusses the role of plant and bacterial hemoglobins (Hbs) in the control of NO levels in nodules.

THE PRODUCTION OF NO IN NODULES

The origin of NO in NFS is still unclear and several NO sources have been evidenced (**Figure 1**). Some studies argue in favor of

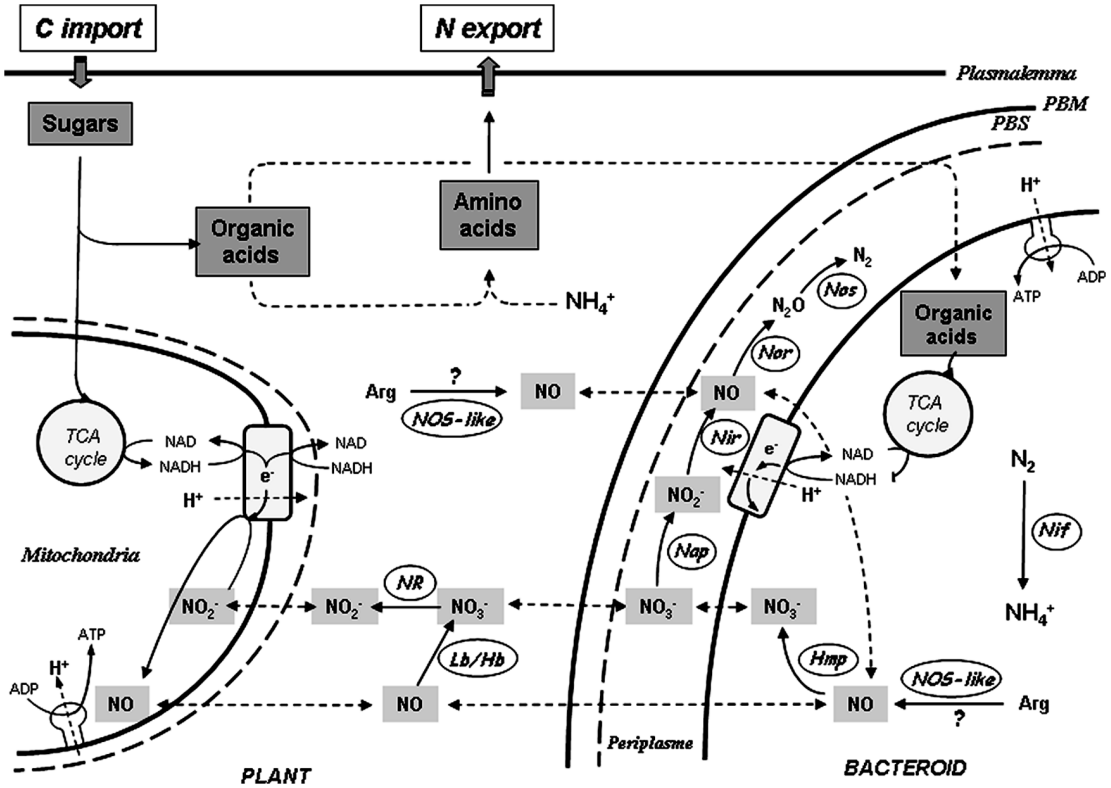

FIGURE 1 | Schematic representation of NO sources in nitrogen-fixing nodules. On the plant side, NO is produced through the cyclic nitrate–NO respiration pathway involving nitrate reductase (NR), mitochondrial electron transfer chain (ETC) and hemoglobin (Lb/Hb). The production of NO via a plant NO synthase-like enzyme (NOS-like) is hypothetical. On the bacteroid side, NO is produced as an intermediate of the denitrification pathway involving nitrate reductase (Nap), nitrite reductase (Nir), NO reductase (Nor), and nitrous oxide reductase (Nos). In the cytosol NO is oxidized into NO_3^- by the flavohemoglobin Hmp. The production of NO via a bacterial NOS-like is hypothetical. In both plant and bacteroid partners, ATP is synthesized due to transmembrane electrochemical gradient generated by proton (H^+) pumping at ETC level. NO_3^-, nitrate; NO_2^-, nitrite; N_2O, nitrous oxide; NH_4^+, ammonium; Arg, arginine; Nif, nitrogenase; PBM, peribacteroid membrane; PBS, peribacteroid space.

the involvement of a NO synthase (NOS)-like enzyme. Thus, in *Lupinus albus* nodule extracts, NO and L-[^{14}C] citrulline were found to be produced in an L-arginine-dependent manner, and the production of L-citrulline was inhibited by a NOS inhibitor (N^{ω}-monomethyl-L-arginine, L-NMMA; Cueto et al., 1996). Baudouin et al. (2006) showed that the addition of L-NMMA in *M. truncatula–S. meliloti* nodule slices impaired NO detection. More recently, the growth and viability of soybean – *Bradyrhizobium japonicum* nodules was found to be negatively affected by the NOS inhibitor N^{ω}-nitro-L-arginine (L-NNA; Leach et al., 2010). However, the molecular identity of such a NOS-like enzyme remains unknown. Using both pharmacological and genetic approaches, Horchani et al. (2011) addressed the role of plant nitrate reductase (NR) and mitochondrial electron transfer chain (ETC) in NO production in *M. truncatula–S. meliloti* nodules. NO production was thus found to be inhibited by tungstate (Tg), a NR inhibitor. In addition, nodules obtained with plant NR RNA-interference (RNAi) double knockdown (MtNR1/2) exhibited reduced NR activities and NO production levels. The reduction of NO production was reversed by nitrite addition, both in the Tg-treated nodules and in MtNR1/2 RNAi nodules, indicating that NO synthesis depends on NR activity, but that NR does not produce NO directly. The inhibition of NO production by ETC inhibitors indicated that mitochondrial ETC was the site of nitrite reduction into NO (Horchani et al., 2011). Thus, in *M. truncatula* nodules, nitrate may be reduced into NO in a two-step mechanism involving successively NR and ETC.

In rhizobia, the denitrification pathway depends on the *napEDABC*, *nirKV*, *norCBQD*, and *nosRZDYFLX* genes that encode NR, nitrite reductase (NiR), NO reductase (Nor), and nitrous oxide (N_2O) reductase, respectively (Bedmar et al., 2005). The expression of the denitrification genes *nirK*, *norC*, and *nosZ* has been reported in soybean – *B. japonicum* functional nodules (Mesa et al., 2004). Using *B. japonicum napA* and *nirK* mutants, it was shown that bacteroid NR and NiR contribute to the main part of NO production, particularly under hypoxic conditions (Meakin et al., 2007; Sanchez et al., 2010). Using a genetic approach, Horchani et al. (2011) similarly showed that around one-third of the NO generated by *M. truncatula–S. meliloti* nodules is produced via the bacteroid denitrification pathway. To date, although a L-arginine-dependent NO synthesis has been reported in free-living *S. meliloti* cells (Pii et al., 2007), such a production was not described in functioning nodules.

NO: THE FOX TO MIND THE GEESE

Nitric oxide concentration was roughly estimated to be in the micromolar range in *Medicago* nodules (Meilhoc et al., 2010), and its level was significantly increased under hypoxic conditions or when nitrate was applied to nodules (Kato et al., 2010; Sanchez et al., 2010; Horchani et al., 2011). NO was first reported to be a potent inhibitor of the *B. japonicum* nitrogenase activity, with a Ki of 56 μM (Trinchant and Rigaud, 1982). The addition of NO donors to *Lotus* and *Alnus firma* nodules, although probably exceeding *in vivo* NO concentrations, led to a reduction in N_2 fixation efficiency (Sasakura et al., 2006; Shimoda et al., 2009; Kato et al., 2010). In this context, *M. truncatula* inoculated with a *S. meliloti hmp* (a bacterial NO-scavenging flavohemoglobin, f-Hb) mutant affected in NO degradation, exhibited a higher NO content in the nodules and a reduced N_2 fixation efficiency as compared to the wild type (WT) strain (Cam et al., 2012). Such effects were indirectly confirmed in *Lotus japonicus* nodules, where the over-expression of non-symbiotic Hb (ns-Hb1, a NO-scavenging enzyme), led to increased N_2 fixation efficiency (Shimoda et al., 2009).

Nitric oxide is known to modify proteins through *S*-nitrosylation, which emerges as a key post-translational modification in plants and a pivotal mechanism to mediate NO bioactivity (Astier et al., 2012). Nitrogenase displays at least three putative *S*-nitrosylation sites (Xue et al., 2010) and, interestingly, different nitrogenase subunits were identified among the *S*-nitrosylated proteins found in *M. truncatula* mature nodules (Puppo et al., 2013). This suggests that NO may inhibit nitrogenase activity through *S*-nitrosylation. Moreover, it was demonstrated that in soybean nodules the NO produced in response to flooding decreased the expression of *B. japonicum nifH* and *nifD* genes encoding the Fe protein and the α-subunit of the MoFe protein of nitrogenase respectively (Sanchez et al., 2010). These observations indicate that at both transcriptional and post-translational levels nitrogenase appears as a primary target for the inhibition of N_2 fixation by NO (**Figure 2**).

Using a *S. meliloti hmp* mutant, Cam et al. (2012) recently showed that an increase in the NO level within the nodule causes its premature senescence, whereas over-expression of *hmp* in nodules leads to a significant delay in nodule senescence, and partly relieves dark-induced senescence of the nodules. These results and others (Cam et al., 2012) provide evidence that NO is produced during aging of legume nodules, and suggest that it could stimulate the senescence of nodules.

NO: A RESPONSE TO HYPOXIA

Based on known adaptation mechanisms of plants to hypoxia, and considering that nodules are microoxic organs, a metabolic role for NO in functioning nodules has been recently proposed (Horchani et al., 2011; Meilhoc et al., 2011). NO production is induced in the roots of plants submitted to hypoxia, and this production is supposed to be linked – via a cyclic respiration process – with improved capacity of the plants to cope with hypoxic stress and to maintain cell energy status (Igamberdiev and Hill, 2009; Gupta and Igamberdiev, 2011). This cyclic respiration, called "nitrate–NO respiration," involves four successive steps (**Figure 1**): (1) the reduction of nitrate to nitrite by NR, (2) the translocation of nitrite from the cytosol into the mitochondria, (3) the reduction of nitrite in NO, via the mitochondrial ETC, allowing respiration and ATP regeneration, and (4) the diffusion of NO from the matrix to the cytosol, where it is oxidized in nitrate by ns-Hb. Thus, under hypoxic conditions, by reducing nitrite to NO, plant mitochondria preserve the capacity to oxidize external NADH, and retain a limited power for ATP synthesis complementing glycolytic ATP production (Gupta and Igamberdiev, 2011).

In functional nodules of *G. max* (Meakin et al., 2007) and *M. truncatula* (Horchani et al., 2011) NO production is increased under hypoxic conditions, and several observations argue in favor of the involvement of nitrate–NO respiration in nodule energy supply. First, plant NR and ETC, and the bacterial denitrification pathway contribute to NO production, via nitrate and nitrite reduction, particularly under hypoxic conditions (Sanchez et al., 2010; Horchani et al., 2011). Second, leghemoglobins (Lbs) and ns-Hb have the capacity to efficiently react with NO to produce nitrate with an elevated rate constant (Herold and Puppo, 2005), and the NO generated at the ETC level may therefore be oxidized into nitrate by Lbs and/or ns-Hbs. Third, the energy status of the nodules depends either partly, or almost entirely, on NR functioning under normoxic, or hypoxic conditions, respectively (Horchani et al., 2011). Thus, in symbiotic nodules a role related to NO metabolism may be fulfilled by Hbs and Hmp in the plant and bacterial partner respectively. The high affinity of these Hbs for NO and their capacity to oxidize NO into nitrate would be favorable to supply the nitrate–NO respiratory cycle in order to maintain a minimal energy status under hypoxia.

On the other hand, during the N_2 fixing process, ammonium generated by bacteroid nitrogenase activity and released in the cytosol of plant cells, is assimilated trough the plant glutamine synthetase (GS1) activity. It has been shown that the *M. truncatula* cytosolic GS1 activity is modulated by NO-mediated tyrosine nitration (Melo et al., 2011). According to the model proposed by the authors, the inhibition of GS1 activity by tyrosine nitration could be directly related to the NO-induced nitrogenase inhibition and the subsequent decrease in ammonium level. Interestingly, a recent analysis of *M. truncatula–S. meliloti* nodules resulted in the identification of about 80 *S*-nitrosylated proteins, such as enzymes of the tricarboxylic acid (TCA) cycle, glycolysis, and N_2 assimilation from either the plant or the bacterial partner (**Figure 2**; Puppo et al., 2013). The activity of some of these enzymes was also found to be inhibited by NO donors (Brouquisse and Castella, unpublished). Considered together, these data suggest that in nodules, NO could also function as a down-regulator of N_2-fixation and carbon metabolism to reduce energy demand under strong hypoxic conditions (**Figure 2**).

ROLE OF HEMOPROTEINS IN THE CONTROL OF NO LEVEL

Toxic, signaling, or metabolic effects of NO depend on its concentration at the site of action (Mur et al., 2012). Thus, in *Lotus japonicus* nodules, high concentrations of NO inhibit N_2 fixation, while low concentrations of NO enhance it (Kato et al., 2010). Therefore, NO steady-state concentration inside nodules should be tightly controlled to limit toxic effects and allow the signaling and metabolic function(s) to occur.

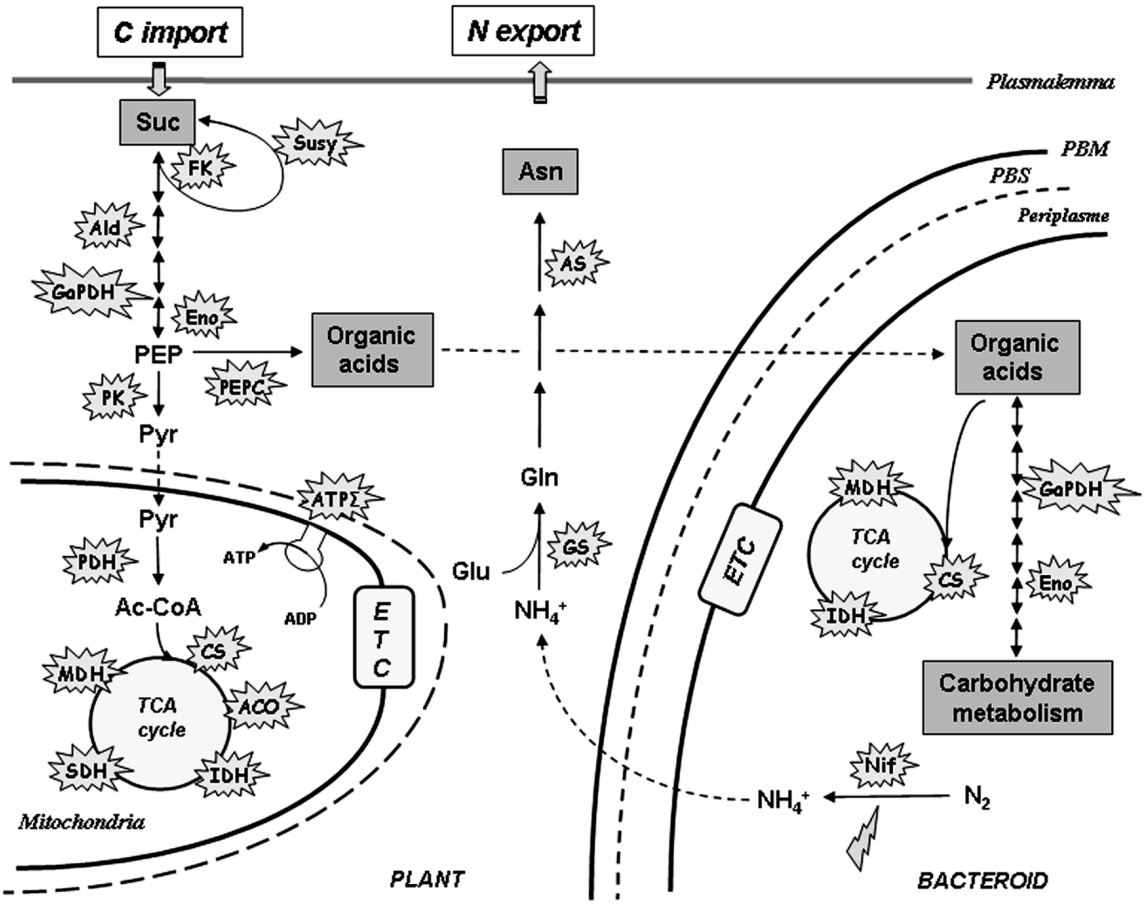

FIGURE 2 | Schematic representation of NO targets in nitrogen, carbon, and energy metabolism in nitrogen-fixing nodules. Explosions refer to enzymes from both plant and bacterial partners found to be either *S*-nitrosylated, or/and inhibited, by NO. Lightning refers to gene repression by NO. Ac-CoA, acetyl-CoA; Asn, asparagine; Glu, glutamate; Gln, glutamine; NH_4^+, ammonium; PEP, phosphoenolpyruvate; Pyr, pyruvate; Suc, sucrose. Aco, aconitase; Ald, aldolase; AS, asparagine synthetase; ATPΣ, ATP synthase; CS, citrate synthase; Eno, enolase; ETC, electron transfer chain; FK, fructokinase; GaPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glutamine synthetase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; Nif, nitrogenase; PEPC, PEP carboxylase; PDH, pyruvate dehydrogenase; PK, pyruvate kinase; SDH, succinate dehydrogenase; Susy, sucrose synthase; PBM, peribacteroid membrane; PBS, peribacteroid space.

Hemoglobins are important proteins known to act as NO storage or scavenger (Gupta et al., 2011). Based on their sequence homology and affinity for oxygen, three families of Hbs have been described in plants: Lbs, ns-Hbs, and truncated Hbs (tr-Hbs; Smagghe et al., 2009; Gupta et al., 2011). The three types of Hbs were reported to be expressed in legumes (Nagata et al., 2008; Bustos-Sanmamed et al., 2011). Lbs accumulate to millimolar concentration in the cytoplasm of infected nodule cells (Appleby, 1992). They are thought to buffer free oxygen in the nanomolar range, avoiding inactivation of nitrogenase while maintaining high oxygen flux for respiration (Ott et al., 2005). Deoxy-Lb was shown to bind NO with a high affinity to form stable complexes in soybean, and it has been proposed that Lb could act as a NO scavenger (Herold and Puppo, 2005). This may also be a function of the ns-Hbs which are ubiquitous in plants (Hill, 2012). Class 1 ns-Hbs could scavenge oxygen traces (Km # 2 nM) to convert NO to nitrate. They were suggested to be responsible for maintaining redox and energy status of plant cells under hypoxia (Igamberdiev and Hill, 2009). NO has been shown to up-regulate ns-Hb expression in a number of plant species. In the actinorhizal symbiosis between *Alnus firma* and *Frankia*, ns-Hb was strongly induced by the application of NO donors and it was shown that *Afns-Hb1*, as a NO scavenger, may support the N_2 fixation ability of members of the genus *Frankia* (Sasakura et al., 2006). Similarly, the overexpression of *ns-Hb1* enhanced symbiotic N_2 fixation in *Lotus japonicus* nodules (Shimoda et al., 2009). tr-Hbs were also shown to be induced in nodules of *M. truncatula* and *Datisca glomerata* (Vieweg et al., 2005; Pawlowski et al., 2007). Based on their expression pattern, it was proposed that they could be involved in NO scavenging. Three classes of Hb have been also described in bacteria: f-Hb (Hmp), single-domain Hb (sd-Hb), and tr-Hb (Sanchez et al., 2011). A bacterial strain of *S. meliloti* mutated in the f-Hb gene (*hmp*) elicited nodules on *M. truncatula* roots with higher levels of NO, lower N_2 fixation efficiency and earlier nodule senescence than the WT (Cam et al., 2012), suggesting that the expression of the Hmp is essential for maintaining NO levels

compatible with symbiosis even though plant Hbs are proficient. In *B. japonicum*, a sd-Hb was also shown to have a NO detoxification role under free-living, microaerobic conditions, suggesting that it could have similar role in nodules during NFS (Sanchez et al., 2011).

Beside Hbs, the respiratory Nor which catalyses reduction of NO into N_2O, is also involved in NO degradation in rhizobia. Thus, in *B. japonicum* inoculated soybean plants subjected to flooding, a significant increase in NO and Lb-NO was observed in *norC* mutant compared with WT nodules (Sanchez et al., 2010). Similarly, NO level was increased in nodules of common bean exposed to nitrate, when elicited by a *R. etli norC* mutant as compared to the WT (Gomez-Hernandez et al., 2011). Interestingly, in *S. meliloti*, NO was found to induce *nor* expression (Meilhoc et al., 2010), and a *nor* mutant strain is more sensitive than a WT strain to a NO donor, and triggers early senescence of *M. truncatula* nodules (Meilhoc et al., 2013). It is important to note that Hmp does not compensate for the absence of Nor, and *vice versa*. On the whole, both plant and bacterial proteins participate in maintaining NO balance in nodules, and although the role of plant Hbs was underlined for years, bacterial NO-degrading enzymes should be considered as major components of this process.

CONCLUSION AND FUTURE ISSUES

The data summarized in this review indicate that NO has dual effects in functioning nodules, inhibiting N_2 fixation, on the one hand, and participating to energy metabolism, on the other hand. It may be considered as a regulator of N_2-fixation and carbon metabolism, by inhibiting nitrogenase and/or enzymes of glycolysis and TCA cycle, to reduce energy demand in stress conditions such as a hypoxic environment. A challenging issue will be to assess precisely how much, where and when NO is produced inside the nodule. Regarding this point, the essential involvement of both plant and bacterial Hbs in the balance of NO level has been particularly evidenced, and much remains to be done to clarify the role of each of these proteins at tissue and cellular level in the functioning nodule.

Another promising issue will be to decipher the role of NO in the perception of oxygen under microoxic conditions. In mammals, a NO-dependent oxygen sensor system was identified, that works through a N-terminal mechanism for protein degradation which is activated by oxygen (Hu et al., 2005). Similar system was recently described in *Arabidopsis* plants, although its NO-dependence was not yet proved (Gibbs et al., 2011; Licausi et al., 2011). Functioning nodules, that are naturally microoxic but metabolically very active organs, appear to be an interesting model to analyze the functioning of such a system, and to investigate the interplay between low oxygen sensing, NO signaling, and metabolic regulation.

Crosstalk between reactive oxygen species (ROS) and NO appears to be a metabolic and signaling key to decipher symbiosis regulation. Peroxynitrite, which is formed when NO reacts with O_2^-, is emerging as a potential signaling molecule to convey NO bioactivity by the selective nitration of Tyr residues in a small number of proteins (Vandelle and Delledonne, 2011). Since both NO and $O_2^{-\bullet}$ are produced in symbiotic nodules (Puppo et al., 2013), it is conceivable that peroxynitrite is formed in these organs. Lb was shown to scavenge peroxynitrite, thus precluding any damaging effect of this species in the nodules (Herold and Puppo, 2005). The recent observation that glutamine synthetase GS1a is nitrated, whereas GS2a is subjected to *S*-nitrosylation in *M. truncatula* nodules (Melo et al., 2011), provides a direct link between NO/$O_2^{-\bullet}$signaling and N_2 metabolism in root nodules. It may be also noted that many of the proteins identified as being *S*-nitrosylated in the symbiotic interaction have also been reported to be *S*-sulfenylated (Oger et al., 2012) suggesting that the same protein may be differentially regulated depending on redox state. The possible regulation of nodule NADPH oxidase activity by NO (Yun et al., 2011; Marino et al., 2012) could be important in the link between NO and O_2^-.

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Current status and proposed roles for nitric oxide as a key mediator of the effects of extracellular nucleotides on plant growth

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Recent data indicate that nucleotides are released into the extracellular matrix during plant cell growth, and that these extracellular nucleotides induce signaling changes that can, in a dose-dependent manner, increase or decrease the cell growth. After activation of a presumed receptor, the earliest signaling change induced by extracellular nucleotides is an increase in the concentration of cytosolic Ca^{2+}, but rapidly following this change is an increase in the cellular level of nitric oxide (NO). In *Arabidopsis*, mutants deficient in nitrate reductase activity (*nia1nia2*) have drastically reduced nitric oxide production and cannot transduce the effects of applied nucleotides into growth changes. Both increased levels of extracellular nucleotides and increased NO production inhibit auxin transport and inhibit growth, and these effects are potentially due to disruption of the localization and/or function of auxin transport facilitators. However, because NO- and auxin-induced signaling pathways can intersect at multiple points, there may be diverse ways by which the induction of NO by extracellular ATP could modulate auxin signaling and thus influence growth. This review will discuss these optional mechanisms and suggest possible regulatory routes based on current experimental data and predictive computational analyses.

Keywords: nitric oxide, extracellular nucleotides, apyrase, auxin transport, post-translational modifications, *S*-nitrosylation, Tyr-nitration, peroxynitrite

INTRODUCTION

For over 40 years scientists have known that the main energy currency of the cell, ATP, is sometimes released by cells into their extracellular matrix (ECM). In the ECM, ATP functions not primarily to drive energy-dependent reactions, but primarily to bind to receptors and activate signaling changes (Khakh and Burnstock, 2009). The data on this topic have come almost exclusively from studies on animals. However, during the last 10 years an increasing number of reports have demonstrated that signaling changes induced by extracellular ATP (eATP) and other nucleoside triphosphates and diphosphates are a common phenomenon also in plants (Tanaka et al., 2010; Clark and Roux, 2011). In animals, these signaling changes are known to begin with the activation of well-characterized receptors, termed purinoceptors, which fall into two main categories: P2X, which are ion-channel linked, and P2Y, which are G-protein linked. In plants, too, there is strong indirect evidence that there may be at least two kinds of plasma-membrane-localized receptors for extracellular nucleotides (Demidchik et al., 2009, 2011), but their primary structures are clearly different from the animal purinoceptors, and as yet their identity is unknown (Clark and Roux, 2011).

The processes that release ATP and other nucleotides into the ECM are similar in plants and animals and include secretion, active transport, and wound or pathogen events that break the plasma membrane or make it leaky (Roux and Steinebrunner, 2007). Animals and plants also use similar enzymes to limit the build-up of eATP, primarily ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases) or ectoapyrases, which remove the terminal phosphate from nucleoside triphosphate diphosphohydrolases (NTPs) and nucleoside diphosphohydrolases (NDPs; Knowles, 2011).

Although the search to identify plant purinoceptors has not yet yielded definitive results, the signaling changes induced by extracellular nucleotides in animals and plants are already known to be remarkably similar. They begin with a rapid increase in the concentration of free cytosolic calcium ($[Ca^{2+}]_{cyt}$; Demidchik et al., 2003, 2009, 2011; Jeter et al., 2004; Burnstock et al., 2010). Afterward, early downstream changes include increased production of superoxide and NO (D'Andrea et al., 2008; Clark and Roux, 2009; Harada, 2010; Tanaka et al., 2010). Sueldo et al. (2010) reported that eATP-induced NO production is downstream of phosphatidic acid production in suspension cultured tomato cells. In plants, mutants that are suppressed in their ability to make either superoxide or nitric oxide are insensitive to the effects of applied nucleotides on cell growth (Clark et al., 2010) and stomatal aperture (Clark et al., 2011; Hao et al., 2012), which suggest these signaling intermediates are needed to convert eATP receptor activation to physiological changes in cells.

This review focuses on the requirement for NO production to transduce extracellular nucleotide signals into growth and other physiological changes in plants. Because there is strong evidence linking extracellular nucleotide effects to changes in auxin transport (Tang et al., 2003; Liu et al., 2012), the question of how

eATP-induced changes in NO production could alter auxin transport becomes especially relevant. As noted by Liu et al. (2012), current evidence favors post-transcriptional events as being key to changing the molecular activities that drive auxin transport, so this review will especially highlight the role of NO in protein modifications that could rapidly alter either the transport or concentration of auxin in cells.

KINETICS OF SIGNALING RESPONSES INDUCED BY EXTRACELLULAR NUCLEOTIDES

Two of the better-documented occasions of ATP release by plant cells are wounding (Song et al., 2006) and cell expansion (Kim et al., 2006; Wu et al., 2007; Clark et al., 2011). Wounding, of course, breaks the plasma membrane and allows the leakage of cytoplasmic ATP (concentration ~mM; Gout et al., 1992) into the ECM. Cell expansion is thought to require the delivery of secretory vesicles to the plasma membrane, and, based on the animal literature, these vesicles can carry up to mM ATP and release it into the ECM upon their fusion with the plasma membrane (Lazarowski et al., 2003). Alternatively, plant cells also release ATP when their membranes are stretched or mechanically stimulated (Jeter et al., 2004; Weerasinghe et al., 2009), and membranes are certainly stretched when plant cells expand. Both wounding and cell expansion would release ATP quickly, and once in the ECM, ATP, and ADP can induce calcium transport changes and thus initiate signal transduction in less than 30 s (Demidchik et al., 2009, 2011).

Downstream of the increase in $[Ca^{2+}]_{cyt}$ induced by extracellular nucleotides are increases in the production of both reactive oxygen species (ROS; e.g., superoxide and H_2O_2; Kim et al., 2006; Song et al., 2006; Tonon et al., 2010; Sun et al., 2012) and nitric oxide (Foresi et al., 2007; Wu and Wu, 2008; Reichler et al., 2009; Clark et al., 2010, 2011). Mutant analyses indicate that both of these changes are needed for the growth and other cellular changes induced by extracellular nucleotides (Clark et al., 2010, 2011). Mutants null for the D/F subunit of NADPH oxidase, which catalyzes superoxide production, or *nia1nia2* double knockouts, which are null for two genes encoding the nitrate reductase enzyme that accounts for significant fraction of the NO production in root hairs (Clark et al., 2010), do not show growth or stomatal aperture responses to applied nucleotides (Reichler et al., 2009; Clark et al., 2010, 2011). Two recently described signaling mechanisms that might link the eATP-induced increase in $[Ca^{2+}]_{cyt}$ and the activation of NADPH oxidase activity should be evaluated; a calmodulin-domain protein kinase (CDPK), which can activate NADPH oxidase by phosphorylation (Yoshioka et al., 2011), and the NO-mediated regulation of NADOH oxidase by *S*-nitrosylation (Yoshioka et al., 2011; Yun et al., 2011). The link between increased $[Ca^{2+}]_{cyt}$ and activation of nitrate reductase is not as well documented, although this enzyme activity may also be regulated by phosphorylation (Lea et al., 2004). Wu and Wu (2008) found that eATPγS must induce an increase $[Ca^{2+}]_{cyt}$ in hairy roots in order to stimulate NO production. Of course, there are other sources of NO production in plants in addition to nitrate reductase, and there is evidence for cross-talk between these enzymes and Ca^{2+} signaling (Besson-Bard et al., 2008).

Given the rapidity of eATP release by cells and the need for NO production to transduce the eATP signal into cellular changes, it is surprising that until now the earliest detection of NO production is 10 min or more after applied nucleotide treatment (Reichler et al., 2009; Clark et al., 2010). This delayed detection may reflect limitations of the assay methods more than actual delay in NO production. Nonetheless, knowing more precisely the kinetics of nucleotide-induced NO production will be important for determining whether NO plays a primary or secondary role in mediating the broad effects of eATP on plant cell growth and physiology, and specifically on auxin transport.

NITRIC OXIDE-DEPENDENT PROTEIN MODIFICATIONS

Two widely studied post-translational modifications (PTMs) that result from an increase in NO are nitration of tyrosine residues generating modified 3-nitrotyrosines, and nitrosylation of cysteine residues (*S*-nitrosylation). Thorough reviews of the proteomic approaches used in plants to identify NO signaling factors (Bykova and Rampitsch, 2013; Jacques et al., 2013; Kovacs and Lindermayr, 2013) and reviews of the various signaling pathways in plants that employ NO-dependent PTM (Astier and Lindermayr, 2012; Corpas et al., 2013) were recently published, so we will not attempt to replicate these. We will focus on what mechanisms link NO-mediated modifications to eATP effects on plant growth generally and auxin transport specifically.

To understand this regulation it will be important to confidently identify which proteins are *S*-nitrosylated or Tyr-nitrated, and then evaluate if these modifications play a central role in growth control. A standard method for detecting *S*-nitrosylation of proteins, called the biotin-switch method, relies on NO donor treatment of samples prior to identification of modified proteins (Jaffrey et al., 2001). *S*-nitrosylation of *Arabidopsis* proteins was detected by this method after NO-donor treatment in cell suspension culture extracts and leaf tissue (Lindermayr et al., 2005). More recently, a modification of this method that does not rely on application of an NO donor was used to identify endogenously *S*-nitrosylated *Arabidopsis* proteins, again from cell culture (Fares et al., 2011). Detailed analysis of specific plant proteins modified by *S*-nitrosylation demonstrates that this modification can regulate protein activity (Astier and Lindermayr, 2012; Feng et al., 2013).

The nitrotyrosine PTM has been experimentally detected in proteins of only a few plant systems to date, including 2-week-old *Arabidopsis* whole seedlings (Lozano-Juste et al., 2011), hypocotyls of 9-day-old sunflowers (Chaki et al., 2009), and pea plants at several different stages of development (Begara-Morales et al., 2013). Each of these studies has demonstrated a regulatory role for the Tyr-nitration observed in at least one protein, as well as identified numerous other targets for this PTM.

Several *Arabidopsis* proteins that have been experimentally shown to be *S*-nitrosylated or Tyr-nitrated (Lindermayr et al., 2005; Fares et al., 2011; Lozano-Juste et al., 2011) may play a role in eATP signaling pathways because they function in auxin transport or signaling, in ROS signaling, or in wall extensibility. Included in the small number of plant proteins whose regulation by NO has been experimentally validated is the auxin receptor TIR1 (Terrile et al., 2012). This finding indicates that the regulation of growth

and development by auxin includes, in at least one case, NO-dependent PTM of a key protein in auxin signaling, and supports the need to evaluate additional players in this signaling pathway as targets for these regulatory modifications.

There is growing evidence for cross-talk between NO and auxin signaling pathways in root growth and morphology and in responses to iron deficiency (Simontacchi et al., 2013). For example, auxin and NO are both implicated in heavy metal stress responses (Peto et al., 2011; Xu et al., 2011; Kolbert et al., 2012) and in the formation of both adventitious and lateral roots (Pagnussat et al., 2003, 2004; Correa-Aragunde et al., 2004; Lanteri et al., 2006, 2008; Guo et al., 2008; Liao et al., 2011; Yadav et al., 2011; Li and Jia, 2013). Increasing NO levels in *Arabidopsis* primary roots results in a decrease of the polar auxin transport mediated by PIN-FORMED 1 (PIN1), and consequent growth inhibition (Fernandez-Marcos et al., 2011). More recently, auxin was suggested to control root morphology by inducing *S*-denitrosylation of an ascorbate peroxidase enzyme involved in redox regulation (Correa-Aragunde et al., 2013). Nitric oxide also plays a role in auxin-induced stomatal opening (She and Song, 2006). Speculatively, this auxin–NO connection could apply also to effects mediated by extracellular nucleotides, as both eATP and apyrase expression influence auxin transport (Tang et al., 2003; Liu et al., 2012), and both regulate stomatal aperture (Clark et al., 2011) and cell growth (Clark et al., 2010) in an NO-dependent manner.

SPECULATION ON POSSIBLE ROLES FOR NITRIC-OXIDE BASED POST-TRANSLATIONAL REGULATION IN eATP RESPONSES

Unraveling the specific steps of any signaling pathway is a complicated task, because signaling typically occurs in a feedback process wherein well-characterized steps such as increased $[Ca^{2+}]_{cyt}$ and protein phosphorylation are the most commonly observed. While studies have shown that both NO-mediated PTMs mentioned above are reversible and can induce physical changes to proteins that regulate their activity (Lindermayr et al., 2005; Lozano-Juste et al., 2011), another interesting possibility that remains to be studied is an indirect regulatory role for these modifications. Bykova

Table 1 | Computationally predicted NO mediated modifications of proteins implicated in eATP signaling (Xue et al., 2010; Liu et al., 2011). Only predictions included in the "high" threshold category are included here (10% FDR).

Gene name	AGI code	*S*-nitrosylation		Tyr-nitration	
		Position	Peptide	Position	Peptide
PLD1, Phospholipase D α 1	AT3G15730	739	LDPSSLE**C**IEKVNRI	77	EPKNPKW**Y**ESFHIYC
				618	LEEDPRN**Y**LTFFCLG
				803	ILGTKSD**Y**LPPILTT
PLD2, Phospholipase D α 2	AT1G52570	211	KNYEPHR**C**WEDIFDA	15	GRLHATI**Y**EVDHLHA
		739	LDPSSQE**C**IQKVNRV	618	EGEDPRD**Y**LTFFCLG
PLC, Phospholipase C	AT5G58670	11	SFKVCFC**C**VRNFKVK	254	STKPPKE**Y**LQTQISK
		226	FGGSLFQ**C**TDETTEC		
GPA1, G-protein α Subunit 1	AT2G26300	5	MGLL**C**SRSRHHT	74	DEGELKS**Y**VPVIHAN
				106	NETDSAK**Y**MLSSESI
AGG1, G-protein γ subunit 1	AT3G63420	54	TDIVSTV**C**EELLSVI	None predicted	
AGG2, G-protein γ subunit 2	AT3G22942	56	MDNASAS**C**KEFLDSV	None predicted	
Apyrase 1	At3g04080	322	SGASLDE**C**RRVAINA	None predicated	
Apyrase 2	At5g18280	None predicted		8	MLNIVGS**Y**PSPAIVT
				410	PLEGEDS**Y**VREMYLK
Apyrase 3	At1g14240	None predicted		473	VVTPNSD**Y**NGKSRKY
				480	YNGKSRK**Y**LGF
Apyrase 4	At1g14230	54	IIFVIVA**C**VTIALGL	11	SGSDEGV**Y**AWVVANH
		342	AAGNFSE**C**RSAAFAM	303	DLSNVAK**Y**KI
Apyrase 5	At1g14250	334	AAGDFTK**C**RSATLAM	None predicted	
Apyrase 6	At2g02970	352	AGGNYSQ**C**RSAALTI	40	APSSSST**Y**TLTKPNS
				349	SFQAGGN**Y**SQCRSAA
				549	YDLEKGR**Y**IVTRIR
Apyrase 7	At4g19180	None predicted		67	SLQDFSS**Y**HGFDPEE

Proteins AGB1 and GCR1 are not included because neither modification was predicated at any residue.
The position of the cysteine or tyrosine amino acid predicted to be modified is given and indicated in bold.

and Rampitsch (2013) suggest that these NO-dependent changes might serve to reversibly occupy an amino acid that could be otherwise modified in a way that would lead to a different activity change (i.e., phosphorylation, carbonylation, or disulfide bond formation).

Predictive, sequence-specific models have been developed to identify sites of cysteine–nitrosylation (Xue et al., 2010) and tyrosine nitration (Liu et al., 2011). These algorithms were developed from K-means clustering methods, and can be used with different thresholds of reliability, high/medium/low, based on the false discovery rate (FDR; 10%/15%/20%). Like efforts to experimentally identify the protein modifications directly, these predictive models are new and still being optimized, but they can be used to identify potential targets of study for these regulatory modifications.

Apyrase proteins serve to regulate extracellular ATP concentration in animal cells (Plesner, 1995; Gaddie and Kirley, 2010), and a similar role may exist for these proteins in plant cells. The *Arabidopsis* nucleoside triphosphate–diphosphohydrolases termed apyrase 1 and 2 have been implicated in e-ATP signaling (Clark et al., 2011; Liu et al., 2012), although they may do so from a Golgi locale (Chiu et al., 2012; Schiller et al., 2012) rather than from a plasma membrane site. When ecto-apyrase activity is inhibited by antibodies raised to APY1 and APY2, the [eATP] of media in which pollen tubes are growing rises several fold and pollen tube growth is inhibited (Wu et al., 2007). Similarly, when APY1/APY2 expression is suppressed by RNAi in R2-4A mutants, this raises the [eATP] of the media and inhibits seedling growth (Salmi, Kim and Roux, unpublished). Although the expression/and or activity of APY1 and 2 appear to influence [eATP], and sites of [eATP] release in roots coincide with sites of increased expression of *APY1* and *APY2* (Roux et al., 2008), it is of course possible that the Golgi function of APY1 and APY2 could regulate growth independent of their influence on [eATP]. Theoretically, other members of the apyrase family could also help regulate [eATP]. At least one Tyr-nitration or *S*-nitrosylation site is predicted in the proteins encoded by each of the seven members of the *Arabidopsis thaliana* family of apyrase genes (**Table 1**; Yang et al., 2013), and these predictions should be experimentally evaluated.

Nitric oxide production is necessary for the cellular response to extracellular nucleotides (Clark et al., 2010, 2011). Similarly, respiratory burst oxidases also play a critical role in mediating plant responses to eATP (Suzuki et al., 2011). Moreover, the timing of

Table 2 | Computationally predicted NO mediated modifications of known auxin transport proteins (Xue et al., 2010; Liu et al., 2011). Only predictions included in the "high" threshold category are included here (10% FDR).

Gene name	AGI code	*S*-nitrosylation		Tyr-nitration	
		Position	Peptide	Position	Peptide
AUX 1	AT2G38120	467	LFAKCYQ**C**KPAAAAA	None predicted	
LAX 1	AT5G01240	216	MHHTKSL**C**LRALVRL	None predicted	
LAX 2	AT2G21050	None predicted		16	ETVVVGN**Y**VEMEKDG
LAX 3	AT1G77690	None predicted		14	ETVVAGN**Y**LEMEREE
PIN 1	AT1G73590	None predicted		18	MTAMVPL**Y**VAMILAY
				480	LIRNPNS**Y**SSLFGIT
PIN 2	AT5G57090	None predicted		18	LAAMVPL**Y**VAMILAY
				335	RSMSGEL**Y**NNNSVPS
				505	LIRNPNT**Y**SSLFGLA
PIN 3	AT1G70940	553	LQPKLIA**C**GNSVATF	498	LIRNPNT**Y**SSLIGLI
PIN 4	AT2G01420	525	LQPKIIA**C**GNSVATF	18	LTAVVPL**Y**VAMILAY
				470	LIRNPNT**Y**SSLIGLI
PIN 7	AT1G23080	425	NGLHKLR**C**NSTAELN	None predicted	
ABCB 1	AT2G36910	1062	ALVGPSG**C**GKSSVIS	743	MIKQIDK**Y**CYLLIGL
				908	EAKIVRL**Y**TANLEPP
				1155	LPEGYKT**Y**VGERGVQ
				1256	KNHPDGI**Y**ARMIQLQ
ABCB 4	AT2G47000	918	IRTVASF**C**AEDKVMN	277	NKHLVTA**Y**KAGVIEG
		1208	QEALDQA**C**SGRTSIV		
ABCB 19	AT3G28860	98	VYLGLVV**C**FSSYAEI	240	QVRTVYS**Y**VGESKAL
		1215	STIRGVD**C**IGVIQDG	595	LIAKSGA**Y**ASLIRFQ
TIR1	AT3G62980	516	RSLWMSS**C**SVSFGAC	450	LTDKVFE**Y**IGTYAKK
		551	PDSRPES**C**PVERVFI		

The position of the cysteine or tyrosine amino acid predicted to be modified is given and indicated in bold.

e-ATP-induced production of NO and ROS is similar (both within ∼30–45 min; Clark et al., 2010, 2011). To the extent that NO and ROS are induced at about the same time and are both needed for plants to respond to eATP, these results suggest the possibility that peroxynitirite could serve to induce Tyr-nitration and thus serve as an important regulator of its own production in a feedback mechanism.

Peroxynitrite ($ONOO^-$) is a potent oxidant and nitrating species that can be formed by the reaction of NO and O_2^- when both of these signaling molecules are present at the same time in the same cell. Plant biologists are just beginning to assay the role of peroxynitrite in plant growth and development (Leitner et al., 2009; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Vandelle and Delledonne, 2011), and thus far it is implicated in hypersensitive defense responses (Saito et al., 2006; Leitner et al., 2009; Gaupels et al., 2011; Bellin et al., 2013) and root development and senescence (Gaupels et al., 2011; Begara-Morales et al., 2013). Peroxynitrite can oxidize proteins and membrane lipids causing cellular damage, and its formation is most likely controlled by the local production of superoxide (Vandelle and Delledonne, 2011). Through this peroxynitrite mechanism, oxidation of lipids may play a role in plant lipid signaling pathways (Sanchez-Calvo et al., 2013).

Could peroxynitrite help mediate some eATP effects? Chivasa et al. (2005) have proposed a role for eATP in programmed cell death, and there is evidence that peroxynitrite may help mediate programmed cell death in plant cells (Serrano et al., 2012a,b). eATP has also been implicated in plant pathogen responses (Chivasa et al., 2009), and a signaling role for $ONOO^-$ in these responses has been documented (Saito et al., 2006; Gaupels et al., 2011). More interesting is the potential role for $ONOO^-$ in plant growth responses to applied eATP. A growth-inhibiting concentration of eATP produces high levels of NO and ROS, while lower, growth-promoting concentrations of eATP induce low levels of NO and ROS (Clark et al., 2010). Both situations may lead to the production of peroxynitrite, which could mediate the growth regulatory effects of eATP.

Another important target for peroxynitrite-mediated nitration in animal cells is the second messenger cyclic guanosine monophosphate (cGMP; Akaike et al., 2010; Sawa et al., 2013). Recently, a role of nitrated cGMP (8-nitro-cGMP) in Abscisic acid (ABA)-induced stomatal closing was discovered (Joudoi et al., 2013). Because ABA-induced stomatal closure can be partially blocked by mammalian purinoceptor antagonists (Clark et al., 2011), and treating *Arabidopsis* leaves with high levels of eATP induces both NO and ROS, it will be important to determine if eATP treatment causes nitration of cGMP in guard cells. Recently, cGMP was shown to promote lateral root formation in *Arabidopsis* by regulating polar auxin transport (Li and Jia, 2013). Thus, a plausible speculation is that nitration of cGMP might also play an important role in regulating auxin transport.

The effects of exogenously applied ATP and ATP analogs are pronounced in root development (Lew and Dearnaley, 2000; Tang et al., 2003; Wu and Wu, 2008; Wu et al., 2008). Proper localization of auxin is necessary for normal root development. In the apyrase mutants described by Liu et al. (2012) localization of several auxin transporters and the abundance of transcripts encoding these transporters were not altered in plants with inhibited auxin transport and stunted and altered root anatomy. One mechanism for this could be regulation of the transporter activity, and NO-mediated PTMs are likely candidates for this regulation. Several proteins known to be involved in polar auxin transport have predicted *S*-nitrosylation and Tyr-nitration sites (**Table 2**), and these predictions should be experimentally evaluated.

CONCLUSION AND FUTURE DIRECTIONS

There is increasing evidence to support a role for NO-mediated PTMs of proteins in the regulation of plant cellular processes by eATP. Although numerous plant proteins have been predicted to undergo these changes and experimentally shown to have them in various conditions, the regulatory role of these PTMs remains to be demonstrated in all but a few cases. Given the central role of auxin in plant growth control, it is likely that the dramatic effects of extracellular nucleotides on auxin transport account for many of their effects on plant growth. Thus, a more complete understanding of how NO regulates auxin transport, whether by PTM of auxin transporters or by other mechanisms, will be key to clarifying why eATP-induced NO production is a necessary step in transducing extracellular nucleotide effects on plant growth and development.

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Nitric oxide, antioxidants and prooxidants in plant defence responses

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In plant cells the free radical nitric oxide (NO) interacts both with anti- as well as prooxidants. This review provides a short survey of the central roles of ascorbate and glutathione—the latter alone or in conjunction with S-nitrosoglutathione reductase—in controlling NO bioavailability. Other major topics include the regulation of antioxidant enzymes by NO and the interplay between NO and reactive oxygen species (ROS). Under stress conditions NO regulates antioxidant enzymes at the level of activity and gene expression, which can cause either enhancement or reduction of the cellular redox status. For instance chronic NO production during salt stress induced the antioxidant system thereby increasing salt tolerance in various plants. In contrast, rapid NO accumulation in response to strong stress stimuli was occasionally linked to inhibition of antioxidant enzymes and a subsequent rise in hydrogen peroxide levels. Moreover, during incompatible *Arabidopsis thaliana-Pseudomonas syringae* interactions ROS burst and cell death progression were shown to be terminated by S-nitrosylation-triggered inhibition of NADPH oxidases, further highlighting the multiple roles of NO during redox-signaling. In chemical reactions between NO and ROS reactive nitrogen species (RNS) arise with characteristics different from their precursors. Recently, peroxynitrite formed by the reaction of NO with superoxide has attracted much attention. We will describe putative functions of this molecule and other NO derivatives in plant cells. Non-symbiotic hemoglobins (nsHb) were proposed to act in NO degradation. Additionally, like other oxidases nsHb is also capable of catalyzing protein nitration through a nitrite- and hydrogen peroxide-dependent process. The physiological significance of the described findings under abiotic and biotic stress conditions will be discussed with a special emphasis on pathogen-induced programmed cell death (PCD).

Keywords: nitric oxide, reactive oxygen species, signaling, peroxynitrite, glutathione, ascorbate, antioxidant system, programmed cell death

INTRODUCTION

Exposure of plants to abiotic and biotic stress can cause a deregulation, over-flow or even disruption of electron transport chains (ETC) in mitochondria and chloroplasts. Under these conditions molecular oxygen (O_2) acts as an electron acceptor giving rise to the accumulation of reactive oxygen species (ROS). Singlet oxygen ($^1O_2$), the hydroxyl radical (OH), the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are all strongly oxidizing compounds and therefore potentially harmful for cell integrity. Among them, H_2O_2 is the most stable ROS being formed in the reaction of $^1O_2$ with O_2^- and as a product of spontaneous dismutation of O_2^- (Foyer and Noctor, 2009).

During evolution, land plants have developed sophisticated measures for controlling ROS levels amongst others by the antioxidant system or—as named after their discoverers—Foyer-Halliwell-Asada cycle (**Figure 1**) (Buchanan et al., 2002; Foyer and Noctor, 2009). Central elements of the system are the two redox couples ascorbate (AsA)/dehydroascorbate (DHA) and glutathione (GSH)/glutathione disulfide (GSSG). In the detoxification part of the antioxidant system superoxide dismutase (SOD) converts O_2^- to O_2 and H_2O_2. The latter then can be degraded by catalase (CAT), ascorbate peroxidase (APX) and several other enzymes (**Figure 1**). In the course of H_2O_2 degradation by APX AsA is oxidized to monodehydroascorbate (MDHA) and DHA. AsA and GSH can also directly be oxidized by ROS, although with slower kinetics. In the regeneration pathway MDHA reductase (MDHAR), DHA reductase (DHAR) and glutathione reductase (GR) recycle the antioxidants from their oxidized back to the reduced form. MDHAR and GR use NADPH as a reducing equivalent whereas DHAR uses GSH (**Figure 1**).

However, apart from being toxic by-products of energy metabolism, ROS have also essential functions in primary and secondary metabolism, development, and stress responses. For instance, H_2O_2 acts as a signal in the regulation of stomatal closure and serves as a substrate of peroxidases during cell wall synthesis and fortification (Neill et al., 2008; O'brien et al., 2012). To date, O_2^- and H_2O_2 are the best studied ROS, mainly because of well-established detection techniques. During signaling processes, ROS arises from the ETC but are also enzymatically produced by various peroxidases and oxidases (Foyer and Noctor, 2009; Mittler et al., 2011). Here, we will assign the term prooxidants for ROS and ROS-producing enzymes and the term antioxidants

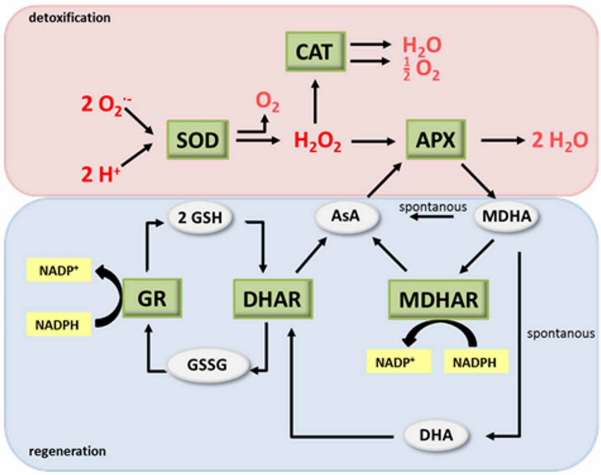

FIGURE 1 | The antioxidant system. (modified after Buchanan et al., 2002). AsA, ascorbate; DHA, dehydroascorbate; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; DHAR, DHA reductase; GR, glutathione reductase; GSH glutathione; GSSG, glutathione disulphide.

for elements of the antioxidant system. During stress signaling, the redox homeostasis of plant cells is tightly controlled. Antioxidants modulate timing and extent of ROS accumulation and additionally function as signals by their own rights. ROS levels increase either by up-regulation of prooxidant enzyme activity, (de−) regulation of electron flow or down-regulation of the antioxidant system. Redox signals are probably transduced by oxidation of proteins such as ROS-activated transcription factors and kinases (Foyer and Noctor, 2009; Mittler et al., 2011). Also other molecules including lipids and fatty acids are modified by ROS with implications for their signaling functions (Farmer and Mueller, 2013).

Similar to ROS, NO is a small redox signal with versatile chemistry. It is a relatively stable radical but rapidly reacts with other radicals including ROS (Hill et al., 2010). Products of these reactions are reactive nitrogen species (RNS) such as the nitrosonium cation (NO^+), the nitroxyl anion (NO^-) and higher oxides of NO including $ONOO^-$, NO_2, and N_2O_3. RNS have chemical properties different from their precursors and may trigger specific physiological responses. Like ROS, NO is an important messenger in many physiological processes. It is a stress signal involved in plant responses to high salt, excess light, cold, heat, ozone, UV-B and various pathogens (Leitner et al., 2009; Gaupels et al., 2011a; Mur et al., 2013). Despite the ever-growing importance of NO in plant research, only little is known about enzymatic sources and molecular receptors of NO. Best characterized is the role of NO in stomatal closure and pathogen defence (Mur et al., 2013). In both processes, NO interacts with H_2O_2 without exact molecular mechanisms deciphered.

The aim of this review is to summarize current knowledge on the interaction of NO with ROS and the antioxidant system in plant stress responses. We will explore how NO can chemically react with pro- and antioxidants and how NO might regulate activity and expression of pro- and antioxidant enzymes. Additionally, functions of non-symbiotic hemoglobins, SOD, GSNOR and peroxiredoxins in regulating RNS homeostasis will be discussed. The last section of this review will detail the roles of individual NO and redox messengers in signaling during stress-induced programmed cell death (PCD).

MANIPULATION OF THE NO LEVEL HAS AN IMPACT ON THE ANTIOXIDANT SYSTEM

The relevance of NO in stress-induced redox signaling was repeatedly investigated by treatment of plants with NO donors before or during exposure to abiotic stress conditions (Hasanuzzaman et al., 2010; Saxena and Shekhawat, 2013). **Table 1** summarizes selected literature reporting the impact of NO donor treatment on H_2O_2 level, antioxidants and activity of antioxidant enzymes in stressed plants. The authors studied 14 different plant species, 11 stressors, and 6 different NO donors providing a comprehensive overview of the current literature on this topic. A common effect of all stress treatments was the accumulation of H_2O_2 often accompanied by an increase in malondialdehyde (MDA) levels pointing to ROS-dependent oxidation of lipids. In 19 of the 23 studies activities of all or at least some of the analyzed antioxidant enzymes were up-regulated. These data suggest that stress causes accumulation of ROS, which may then trigger enhancement of the antioxidant defence system.

Most of the published studies demonstrated accumulation of NO under stress conditions (Hasanuzzaman et al., 2010; Saxena and Shekhawat, 2013). However, results given in **Table 1** as well as other data imply that NO cannot be considered to be a general stress signal. For instance, comparing the effect of 25 μM arsenic between two studies, NO production was induced in *Festuca arundinaceae* but decreased in *Oryza sativa* (**Table 1**) (Singh et al., 2009; Jin et al., 2010). During plant responses to cadmium stress, NO was increased or decreased acting as inducer or inhibitor of stress tolerance, depending on plant species and experimental setup (Arasimowicz-Jelonek et al., 2011a). Moreover, iron deficiency triggered NO signaling in *Arabidopsis thaliana* (Chen et al., 2010) but repressed basal NO synthesis in *Zea mays* (**Table 1**) (Kumar et al., 2010). In this context it is interesting that recent studies revealed NO being a modulator rather than an essential signal in the adaptation of *A. thaliana* to iron deficiency (Meiser et al., 2011). Together, these findings demonstrate that the link between stress perception and NO signaling is seemingly rather indirect whereas stress can directly cause ROS accumulation by disturbing the mitochondrial and plastidic ETC. Further studies are needed for investigating the biological background of the observed species-specific differences in NO regulation under stress conditions. In sum, the above findings support the notion that endogenous NO is often but not always involved in stress tolerance.

Exogenous NO always improved abiotic stress tolerance concomitant with a decrease in H_2O_2 and MDA levels (**Table 1**). This held true, even when endogenous NO was down-regulated, implying that the tested NO donors do not necessarily mimic functions of NO under natural conditions. In the displayed 23 studies, NO treatments either reversed the stress-induced decline

Table 1 | NO donors induce stress tolerance by effecting on the antioxidant properties of plant tissues.

Stress[a]	NO donor	Plant species	Stress effect[c]			Impact of NO donor treatment on abiotic stress-induced changes in antioxidant properties[d]										References
			H_2O_2	NO	Antiox. enz.	H_2O_2	MDA	Glutathione	Ascorbate	SOD	CAT	APX	DHAR	MDHAR	GR	
50 mM NaCl	0.05 mM SNP	*Hordeum vulgare*	+		+	↓	↓			↑	↑	↑				Li et al., 2008
150, 300 mM NaCl	1 mM SNP	*Triticum aestivum*	+		=	↓	↓	↑			↑	↔	↑	↑	↑	Hasanuzzaman et al., 2011
150 mM NaCl	0.2 mM SNP	*Brassica juncea*	+		+	↓	↓			↓	↓	↓			↑	Khan et al., 2012
150 mM NaCl	0.01 mM DETA/NO	*Zea mays*	+	+	+	↓	↓	↑	↑			↑	↑		↑	Keyster et al., 2012
100 mM NaCl	0.2 mM SNP	*Cicer arietinum*			+		↓			↓	↑	↑	↔		↓	Sheokand et al., 2008
Drought (less water)	0.05–0.15 mM SNP	*Oryza sativa*	+		−	↓	↓			↑	↑	↑				Farooq et al., 2009
Drought (10% PEG)	0.1 mM SNP	*Zea mays*	+		+	↓				↑[e]		↑			↑	Sang et al., 2007
Drought (15% PEG)	0.2 mM SNP	*Triticum aestivum*	+		+	↓	↓			↑	↔					Tian and Lei, 2006
0.25, 0.5 mM arsenic	0.25 mM SNP	*Triticum aestivum*	+		±	↓	↑	↑	↑		↑	↔	↑	↑	↑	Hasanuzzaman and Fujita, 2013
0.025 mM arsenic	0.1 mM SNP	*Festuca arundinacea*	+	+	+	↓	↓			↑	↑	↑				Jin et al., 2010
0.025, 0.05 mM arsenic	0.05 mM SNP	*Oryza sativa*	+	−	+	↓	↓			↓	↓	↓				Singh et al., 2009
0.050 mM copper	0.1 mM SNP	*Panax ginseng*	+	+	−	↓	↓	↑	↔	↑	↑	↑			↑	Tewari et al., 2008
0.025 mM cadmium	5 mM SNP	*Brassica juncea*	+	+	+		↓	↓		↓	↓	↓				Verma et al., 2013
5 mM cadmium	0.1 mM SNP	*Oryza sativa*	+		+	↓	↓	↑	↑	↓	↓	↓			↓	Hsu and Kao, 2007
0.5 mM cadmium	0.1 mM SNP	*Helianthus annuus*			±		↓	↑	↓	↓	↓				↓	Laspina et al., 2005
0.01 mM Fe-EDTA	0.01, 0.1 mM SNP	*Zea mays*	+	−	−	↓	↑	↓		↓	↑	↑				Kumar et al., 2010
0.025 mM paraquat	SIN-1, Asc/$NaNO_2$[b]	*Oryza sativa*			+		↓			↑	↑				↑	Hung et al., 2002
0.8–4 mg L^{-1} diquat	0.1 mM SNP	*Solanum tuberosum*	+		±	↓	↓			↔	↑					Beligni and Lamattina, 2002
15 μmol m^{-2} s^{-1} UV-B	0.1 mM SNP	*Phaseolus vulgaris*	+		+	↓		↑		↑	↑	↑				Shi et al., 2005
0.6W m^{-2} s^{-1} UV-B	1 mM SNP	*Spirulina platensis*		+	+		↓			↑	↑					Xue et al., 2007
High light	0.1 mM SNP	*Festuca arundinacea*	+		+	↓	↓			↑	↑	↑			↑	Xu et al., 2010
Desiccation of seeds	100 ppm NO gas	*Antiaris toxicaria*	+		+	↓						↑	↑	↑	↑	Bai et al., 2011
Chilling of seeds	0.03 mM NOC-18	*Baccaurea ramiflora*	+		+	↓		↑				↑	↑	↑	↑	Bai et al., 2012

[a] *Drought stress was induced either by reduced watering or treatment with polyethylene glycol (PEG). 0.01 mM Fe-EDTA causes iron deficiency. Paraquat and diquat are herbicides.*

[b] *0.1 mM SIN-1 or 0.1mM ascorbate (Asc)/0.2 mM $NaNO_2$ was used as NO donors.*

[c] *Stress-induced changes in H_2O_2 and NO levels as well as antioxidant enzyme activities (general tendency).*

[d] *Comparison of combined stress and NO treatment with stress alone treatment. Metabolites are high-lighted. All other parameters represent enzyme activities.*

[e] *Regulation of chloroplastic SOD activity; cytosolic SOD was not influenced by NO donor treatment.*

Arrows indicate up-, down- or no regulation. +,−, ± and = indicate up-, down-, differential- or no regulation.

or even further amplified up-regulation of the antioxidant system. NO donors never caused a down-regulation of antioxidant enzymes as compared to untreated control plants. For instance, salt stress stimulated SOD, CAT, and APX activities, and this effect was enhanced by SNP co-treatment, whereas copper uptake repressed the same enzymes in *Panax ginseng*, which was prevented by SNP (**Table 1**) (Li et al., 2008; Tewari et al., 2008). Again the same enzyme activities were enhanced after arsenic poisoning of *O. sativa* but SNP application prevented this stress effect (**Table 1**) (Singh et al., 2009). These findings were explained by NO acting either (I) as a direct scavenger of ROS or (II) inducer of the antioxidant system. In the first case NO would take over functions of the antioxidant system and thereby prevent its activation, like e.g. in arsenic-exposed rice as described above. In the second case NO would trigger antioxidant gene expression or activate antioxidant enzymes e.g., by posttranslational modifications. Previously, NO donors were reported to repress antioxidant enzyme activities. Particularly, SNP inhibited APX and CAT, decreased GSH/GSSG ratio and induced PCD in Arabidopsis suspension cultured cells (Murgia et al., 2004a). However, the research summarized in **Table 1** was focussed on investigating mechanisms of NO-mediated stress tolerance. Therefore, NO donors were probably applied in such a way as to prevent any severe stress or damage to the plants although sometimes up to 5 mM SNP was used. We will discuss later in this review the dose dependent effects of NO on the antioxidant system and cell death initiation.

A direct chemical interaction of NO with ROS is only possible if cells or plant parts are being loaded with active NO donor solution from start of the stress treatment until sampling as was the case for *Spirulina platensis* cells exposed to UV-B and SNP and *Brassica junceae* leaf discs incubated in salt and DETA/NO donors (**Table 1**) (Xue et al., 2007; Khan et al., 2012). In other studies, however, measurements were done after NO donors were exhausted suggesting that NO released from the donor did not have a direct influence on ROS levels but might be rather involved in the induction of signaling events controlling the cellular redox status. Farooq et al. (2010) reported that imbibition of seeds in SNP solution rendered adult rice plants more tolerant to drought stress. Hence, NO pre-treatment could induce a primed state, which prepares plants to respond more efficiently to future stress episodes (Conrath, 2011). Alternatively, NO treatment itself could impose stress to the plants acting as the priming stimulus. Exogenous NO might also induce synthesis of endogenous NO, which then can exert signaling or scavenger functions even long after the NO donor is exhausted.

NO donors can have undesired side-effects on the plant's physiology. Therefore, NO accumulating transgenic and mutant plant lines were used for assessing the involvement of NO in development and stress signaling. Transgenic *Nicotiana tabacum* and *A. thaliana* expressing the rat neuronal nitric oxide synthase (NOS) behind a 35S promoter accumulated high levels of NO concomitant with developmental defects and altered stress resistance (Chun et al., 2012; Shi et al., 2012). 35S::nNOS lines of Arabidopsis constitutively expressed pathogenesis related (PR) genes, which correlated with enhanced pathogen resistance toward virulent *Pseudomonas syringae DC3000* (Shi et al., 2012). These plants also had improved salt and drought tolerance due to reduced stomatal aperture, and were delayed in flowering. The H_2O_2 content was not determined, but MDA levels were found to be lowered. By comparison, nNOS-expressing tobacco showed growth retardation and constitutive inhibition of CAT, which caused an increase in H_2O_2 levels (Chun et al., 2012). Probably as a consequence of high NO and H_2O_2 levels, these plants developed spontaneous lesions, strongly elevated salicylic acid (SA) levels and PR gene expression. Reduced growth, increased oxidative stress and spontaneous lesions was not observed in nNOS expressing *A. thaliana* plants indicating that they either were less sensitive to NO or accumulated lower levels of NO than the corresponding tobacco transgenic lines.

Collectively, the discussed research argues for ROS being a general stress signal whereas NO signaling depends on the plant species and stress conditions investigated. It can be speculated that NO or the interaction between ROS and NO adds some degree of specificity to the stress signaling by ROS alone. Treatment of plants with NO donors caused a decrease in stress-induced ROS levels and a concomitant enhancement of abiotic stress tolerance. In this process NO might act as a scavenger of ROS or as a signal stimulating the antioxidant potential and/or a primed state of stress defence. Interpretation of the data is complicated by the fact that most of the studies are rather descriptive without exploring the underlying signaling cascades. Moreover, the biological significance of some observed weak effects of NO on ROS and the antioxidant system is ambiguous because slight changes in the cellular redox status could be just a stress marker.

SOURCES AND CELLULAR LOCALIZATION OF NO AND ROS PRODUCTION

NO and certain ROS cooperate in stress signaling, which is partly independent of their respective production sites because both molecules are supposed to be mobile intra- as well as intercellularly (Foyer and Noctor, 2009; Frohlich and Durner, 2011). Therefore, apoplastic sources can contribute to NO and ROS signal transduction within the cell (**Table 2**). Important ROS producing enzymes are the members of the NADPH oxidase family (NOX or Respiratory burst oxidase homolog, RBOH). These plasma membrane-associated enzymes synthesize O_2^- in the apoplast through transfer of electrons from NADPH to molecular oxygen (Mittler et al., 2011). A rapid ROS burst, frequently observed during plant responses to pathogen infection, is usually mediated by the NOX isoforms D and F (Torres et al., 2002). Further oxidases and cell wall-associated peroxidases are present in the apoplast but their roles in stress responses are less well-defined. In comparison to ROS only little is known about NO formation in the extracellular space (**Table 2**). At the acidic pH of the apoplast exogenous NO_2^- was non-enzymatically reduced to NO, which was accelerated by AsA and phenolics (Bethke et al., 2004). The pathway has been investigated in the barley aleuron layer but might occur also in other tissues. A stress-induced NO burst derived from this spontaneous reaction seems only feasible if NO_2^- levels could be rapidly up-regulated, which has not been observed so far. NO_2^- could also be reduced to NO by a membrane-associated nitrite:NO reductase (NiNOR) as described for tobacco (Stöhr et al., 2001). However, NiNOR

Table 2 | Localization of NO and ROS sources in plant cells.

	NO sources	ROS sources
Chloroplast	Nitric oxide synthase–like activity Photosynthetic ETC dependent nitrite reduction	Photosynthetic ETC –ROS production at photosystem I & II $^1O_2$production by triplet state chlorophyll
Peroxisome	Nitric oxide synthase–like activity *Nitrite reduction by xanthine oxidoreductase*	Photorespiration Fatty acid β-oxidation Xanthine oxidase Flavin oxidase
Mitochondria	Respiratory ETC dependent nitrite reduction *Nitric oxide synthase–like activity*	Respiratory ETC –ROS production at complexI, II & III
Cytoplasma	Nitrite reduction by nitrate reductase	Plasma membrane associated quinone oxidase
Apoplast	Spontanous nitrite reduction at acidic pH Plasma-membrane bound nitrite reductase (root specific–NO release to apoplast) *Polyamineoxidase*	Plasma membrane associated NADPH oxidase (ROS release into apolast) Cell wall associated peroxidase Amine oxidase Oxalate oxidase

ETC, electron transport chain. NO sources under debate are given in italics.

cannot be considered a major player in NO signaling because it is exclusively present in roots functioning in the regulation of NO_3^- uptake. Copper amine oxidase 1 (CuAO1) is another candidate enzyme involved in NO synthesis (Wimalasekera et al., 2011). The *A. thaliana* cuao1 mutant is impaired in polyamine- and abscisic acid-induced NO production. The molecular background underlying this interesting phenotype is still unknown.

Cellular compartments simultaneously producing NO and ROS might be focal points of stress signaling (**Table 2**). While chloroplasts and mitochondria are major sources of ROS from photosynthetic and respiratory ETC these organelles are also capable of NO synthesis, one proposed mechanism being the transfer of electrons from the ETCs to NO_2^- by a nitrite: NO-reductase activity. Such ETC-dependent NO formation was observed in isolated choroplasts from tobacco supplied with 25–100 μM NO_2^- and in mitochondria of tobacco suspension cells under anoxia (Planchet et al., 2005; Jasid et al., 2006). More work is needed for investigating if this pathway is active also in stress responses under normoxic conditions. Mammalian NOS oxidizes arginine to citrulline and NO. Although NOS-like activity is considered the most important source of NO accumulation in plant reactions to various stresses the corresponding plant NOS still awaits identification (Leitner et al., 2009; Mur et al., 2013). Recent publications reported on the detection of a NOS-like activity in chloroplasts (Jasid et al., 2006; Tewari et al., 2013). In *A. thaliana* and *Brassica napus* protoplasts NO generation was highest immediately after the isolation procedure and decreased during culture. Experiments with a NOS activity assay and specific enzyme inhibitors suggested that NO originated from a NOS-like source. Moreover, simultaneous accumulation of NO and ROS resulted in the formation of $ONOO^-$ as detected by the fluorescent dye aminophenyl fluorescein (APF) (Tewari et al., 2013). In line with this, treatment with the fungal elicitor cryptogein also triggered rapid accumulation of both NO and ROS in tobacco epidermal cells (Foissner et al., 2000). The above data imply that stress induces the accumulation of ROS and RNS in the chloroplast, which could then locally effect on photosynthesis or diffuse out of the chloroplast to other cellular compartments.

To date, there is no convincing proof of NOS-like activity in mitochondria (**Table 2**; Gupta et al., 2011). In contrast, peroxisomes are a source of NO both during salt stress as well as developmental processes such as lateral root growth (Corpas et al., 2009; Schlicht et al., 2013). In *A. thaliana* transgenic lines expressing GFP linked to peroxisomal targeting signal 1 (PTS1) fluorescence of the NO-specific dye diaminorhodamine co-localized with GFP fluorescence in the peroxisomes. Isolated peroxisomes displayed NOS-like activity, which was calcium dependent and could be inhibited by NOS inhibitors (**Table 2**). 100 mM NaCl stimulated NO synthesis in peroxisomes, which spread into the cytosol, where it probably contributed to $ONOO^-$ formation and protein tyrosine nitration (Corpas et al., 2009). Peroxisomes are active sites of ROS scavenging as well as formation. The main function of peroxisomes is the removal of ROS originating from photosynthetic and mitochondrial ETCs. For this purpose, peroxisomes contain large amounts of CAT but also APX and other antioxidant enzymes. However, after a stress stimulus antioxidant

enzymes can be down-regulated possibly by S-nitrosylation or nitration rendering peroxisomes a ROS source rather than a sink (Sandalio et al., 2013). Peroxisomes are often closely associated with mitochondria and/or chloroplasts. Such functional units are essential for efficient ROS scavenging but it can be speculated that they also represent "reaction vessels" for enhancing ROS/RNS signal interaction.

In the past, microscopic studies with NO-specific dyes suggested higher stress-induced NO accumulation in chloroplasts and peroxisomes than in the cytoplasm (e.g., Foissner et al., 2000; Gaupels et al., 2008; Corpas et al., 2009). One possible explanation for this finding would be that the cytoplasm has a rather low capacity of NO synthesis. While NOS-like activity was not detected, nitrate reductase (NR) is the only confirmed NO source in the cytoplasm (**Table 2**). However, under normal growth conditions NR preferably reduces NO_3^- to NO_2^-, which is then further reduced by nitrite reductase to NH_4^+. Only under special conditions such as anoxia when NO_2^- reaches high levels NR reduces NO_2^- to NO at considerable rates (Gupta et al., 2011; Mur et al., 2013). For this reason, it seems unlikely that NR significantly contributes to rapid stress signaling by NO. Overall, chloroplasts and peroxisomes are probably the most important sources of NO and ROS during stress responses. Available data indicate that both signal molecules are produced simultaneously giving rise to the formation of RNS such as $ONOO^-$. ROS mainly originated from NADPH oxidases and ETCs. The NO burst was driven by a yet unidentified NOS-like activity in chloroplasts and peroxisomes. Nitrite reduction to NO either non-enzymatically or by various reductases is thought to contribute comparably less to the NO burst.

INTERACTIONS BETWEEN NO AND ROS

Chemical interactions between NO and ROS influence concentration, composition and signaling functions of both reaction partners. For instance, H_2O_2 was proposed to react with NO yielding $^1O_2$ and NO^- *in vitro* (Noronha-Dutra et al., 1993). If this chemical pathway occurs *in vivo* is still ambiguous since NO is a rather stable radical, which does not easily bind non-radical species such as H_2O_2. Physiologically more significant is the fusion of NO with O_2^- to give $ONOO^-$ (**Table 3**) (Hill et al., 2010). This radical-radical reaction has a high rate constant and is favored instead of O_2^- dismutation to H_2O_2. As a result, highly cytotoxic and long-lived ROS are replaced by $ONOO^-$, which is short-lived in the cellular environment (Pryor et al., 2006). The exact pathway of $ONOO^-$ and ONOOH (peroxynitrous acid) decay to NO_2^- and NO_3^- at neutral pH is still debated (**Table 3**). It was suggested that ONOOH isomerises to NO_3^- and H^+ either directly or indirectly via the radical intermediates NO_2 and OH (Goldstein and Merenyi, 2008; Koppenol et al., 2012). The peroxynitrite anion on the other hand yields the RNS NO_2, NO, and N_2O_3 during its degradation to NO_2^- (Goldstein and Merenyi, 2008). At neutral pH $ONOO^-$ and ONOOH are both present in cells and together form peroxynitrate (O_2NOO^-/O_2NOOH), which decays to NO_2^- and O_2 as well as $^1O_2$ and NO^- (Khan et al., 2000; Jourd'heuil et al., 2001; Gupta et al., 2009; Miyamoto et al., 2009). Meanwhile it is widely accepted that CO_2 is an important modulator of $ONOO^-$ chemistry in cells. The atmospheric gas rapidly reacts with $ONOO^-$ resulting in NO_3^- and the radicals NO_2 and CO_3^- (carbonate anion radical Bonini et al., 1999; Pryor et al., 2006).

Table 3 | Reaction stoichiometry between ROS and RNS.

ROS	RNS
Hydrogen peroxide: H_2O_2	Nitric oxide: NO
Superoxide: O_2^-	Peroxynitrite: $ONOO^-$
Singlet oxygen: $^1O_2$	Peroxynitrous acid: ONOOH
Hydroxyl radical: OH	Peroxynitrate: O_2NOO^-
Oxygen: O_2	Peroxynitric acid: O_2NOOH
	Nitrosonium cation: NO^+
	Nitroxyl anion:NO^-
	Nitrogen dioxide: NO_2
	Dinitrogentrioxide: N_2O_3
	Nitrosoglutathione: GSNO

REACTION STOICHIOMETRY	References
$NO_2^- + 2\ H^+ \leftrightarrow NO + H_2O$	Pryor et al., 2006
$NO^+ + H_2O_2 \rightarrow ONOO^- + 2\ H^+$	Beligni and Lamattina, 2002
$NO + O_2^- \rightarrow ONOO^-$	Miyamoto et al., 2009
$2\ NO + O_2 \rightarrow 2\ NO_2$	Moller et al., 2007
$NO_2 + NO \leftrightarrow N_2O_3$	Moller et al., 2007
$N_2O_3 + H_2O \rightarrow 2\ NO_2^- + 2\ H^+$	Moller et al., 2007
$ONOOH \rightarrow ONOO^- + H^+$ (Ionisation)	Koppenol et al., 2012
$ONOOH \rightarrow NO_3^- + H^+$ (Isomerisation)	Koppenol et al., 2012
$ONOOH \rightarrow NO_2 + HO$ (Homolysis)	Koppenol et al., 2012
$ONOO^- \rightarrow NO + O_2^-$ (Homolysis)	Koppenol et al., 2012
$O_2NOO^- \leftrightarrow NO_2 + O_2^-$ (Homolysis)	Gupta et al., 2009
$ONOOH + ONOO^- \rightarrow O_2NOO^- + NO_2^- + H^+$	Gupta et al., 2009
$CO_2 + ONOO^- \rightarrow CO_3^- + NO_2$	Pryor et al., 2006

High levels of NO can react with O_2 giving rise to the NO_2 radical (**Table 3**). This pathway is slow in the cytosol but might be efficient in membrane-rich cellular compartments such as chloroplasts and mitochondria owing to the lipophilic nature of NO and O_2 (Liu et al., 1998; Pryor et al., 2006). Under continuous NO production NO_2 will further react to N_2O_3 (Pryor et al., 2006; Moller et al., 2007). All reactive nitrogen oxides decompose to the stable derivatives NO_2^- and NO_3^- within cells. However, as described in the previous section, under acidic conditions e.g., in macrophages and in the plant apoplast N_2O_3, NO, and NO^+ can also originate from NO_2^- upon enzymatic or non-enzymatic reduction (**Table 3**) (Pryor et al., 2006; Combet et al., 2010; Frohlich and Durner, 2011). Hence, dependent on the prevailing cellular environment NO and ROS can interact resulting in the formation of intermediates with distinct molecular properties. For instance, NO, NO^-, NO^+, and N_2O_3 bind to nucleophilic residues of proteins causing nitrosation (covalently bound nitroso/-NO adduct) and cysteine- as well as metal S-nitrosylation (coordinate nitrosyl/··NO adduct) (Hill et al., 2010; Fukuto and Carrington, 2011). In contrast, $ONOO^-$ and the NO_2 radical are involved in oxidation and nitration (covalently bound nitro/-NO_2 adduct) of proteins the best studied modifications being 3-nitro-tyrosine residues (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Gaupels et al., 2011a;

Radi, 2013). NO_2 has less nitrating power than $ONOO^-$ except with protein radicals, which result from the reaction of proteins with ROS or CO_3^- radicals (Bonini et al., 1999; Pryor et al., 2006). To date, the CO_3^- catalyzed binding of NO_2 to tyrosyl residues is thought to be the major route of protein nitration.

NO-dependent protein modifications are reversible, which is important for efficient recovery of NO receptors during stress signaling. In mammalian cells, thioredoxins (TRX) denitrosylate proteins (Tada et al., 2008; Benhar et al., 2009). Recently, the central redox switch NPR1 was suggested to be denitrosylated by TRX-h-3 and -5 during incompatible *A. thaliana/P. syringae* interactions, which caused its monomerisation from oligomers, transfer into the nucleus and subsequent induction of PR genes (Tada et al., 2008). However, the exact mechanism of NPR1 regulation by S-nitrosylation and TRX is still debated (Lindermayr et al., 2010). Denitration of proteins in *A. thaliana* is probably mediated by peptide methionine sulfoxide reductase (PMSR) under normal growth conditions since *pmsr2-1* mutants displayed elevated protein nitration in the night (Bechtold et al., 2009). This enzyme reduces oxidized protein methionine residues using TRX as a co-substrate but how it can function as a denitratase is not yet resolved. Future research will uncover if additional reductases, peroxiredoxin oxidases and peroxidases such as TRX peroxidase are involved in stress signaling by NO-dependent protein modifications.

Apart from proteins many other molecules can be nitrated including lipids, fatty acids, amino acids and nucleotides (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). Recently, 8-nitro-cGMP was uncovered as a down-stream signal of ABA, NO, and ROS in inducing stomatal closure at daytime, whereas cGMP regulated stomatal opening at night (Joudoi et al., 2013). 8-nitro-cGMP is now a prime example of how NO, ROS, and cGMP can be integrated in one signaling cascade triggering a physical response.

NO AND ROS INFLUENCE EACH OTHER'S BIOSYNTHESIS AND DEGRADATION

ROS are well-known inducers of NO synthesis in various plant species, plant parts and tissues. For example, treatment with 100 μM H_2O_2 triggered NO synthesis in roots of *A. thaliana*, which was used in a screen for identification of mutants defective in NO accumulation. This way, the prohibitin PHB3 was uncovered as a regulatory element of ABA- and auxin-induced NO signaling (Wang et al., 2010). Moreover, H_2O_2 elicited a rapid NO burst in guard cells of mung bean leaves (*Phaseolus aureus*) (Lum et al., 2002) as well as NOS activity along with PCD in tobacco BY-2 cells (De Pinto et al., 2006). The interplay between ROS, NO and the antioxidant system will be discussed in more detail in the last section of this review. Exposure to ozone (O_3) led to high ROS levels and rapid NO production in the leaves of *A. thaliana* plants (Ahlfors et al., 2009). During the O_3 response NO acted as a signal in the onset of the hypersensitive response (HR) and in the regulation of defence-related genes thereby interacting with jasmonic acid (JA), ethylene and SA. In the phloem of *Vicia faba* NO accumulation upon treatment with 10 and 100 μM H_2O_2 was dependent on Ca^{2+} and NOS-like enzyme activity (Gaupels et al., 2008). Although induction of NO biosynthesis through H_2O_2 and Ca^{2+} is widely accepted, exact signaling cascades and enzymatic sources of NO are still not well-understood. Effects of H_2O_2 on NO scavenging enzymes such as GSNOR and hemoglobins were not yet investigated.

NO is not just a down-stream signal of H_2O_2 but was also reported to influence ROS production and degradation, which hints at complex feed-back regulation between both signal molecules. NO limits ROS accumulation for instance by inhibition of the ROS producing enzyme NADPH oxidase (Yun et al., 2011). After infection of *A. thaliana* with avirulent pathogens the elevated SNO content inhibited the NADPH oxidase isoform AtRBOHD by S-nitrosylation at Cys 890. According to the author's hypothesis this regulatory process constrains ROS accumulation and subsequent cell death progression (Yun et al., 2011). A means of enhancing antioxidant enzyme activities is the induction of the corresponding genes by NO. Accordingly, 2D-electrophoresis and Western blot analyses revealed that pretreatment with the NO donor SNAP further increased the Al^{3+}-induced protein levels and activities of APX, SOD, and GR, whereas NOS inhibitor and cPTIO suppressed both the Al^{3+} and the SNAP effect (Yang et al., 2013). Alternatively, NO could directly modify protein functions. In *Antiaris toxicaria* NO fumigation improved desiccation tolerance of recalcitrant seeds, which correlated with a decrease in H_2O_2 levels. The authors proposed that S-nitrosylation enhanced the activities of the antioxidant enzymes GR, APX, and DHAR by preventing their oxidation/carbonylation during desiccation (Bai et al., 2011). Moreover, in salt stressed *B. juncea* S-nitrosylation of a Fe-SOD caused an increase in its enzyme activity (Sehrawat et al., 2013).

More commonly, however, NO was associated with inhibition rather than activation of antioxidant enzymes. *In vitro*, tobacco APX and CAT were reversibly inhibited by GSNO, SNAP, and NOC-9 but irreversibly inactivated by SIN-1 (Clark et al., 2000). Inhibition of APX and CAT by NO donors was confirmed in isolated pea mitochondria, leaves of *Pelargonium peltatum* and suspension cultured cells of *A. thaliana* and *N. tabacum* (Murgia et al., 2004a; Arasimowicz-Jelonek et al., 2011b; Marti et al., 2013). SNP and SNAP were the most effective NO donors, whereas GSNO produced variable results. The chemical properties of the donors is an important issue because SNP releases NO^+ and SIN-1 simultaneously O_2^- and NO whereas most other donors deliver NO. Thus, dependent on the NO donor used and the prevailing redox conditions antioxidant enzyme activity could be affected due to oxidation, S-nitrosylation, nitrosation or nitration. Unfortunately, NO- and ROS-dependent protein modifications were not investigated in the above studies.

Any of the enzymes APX, SOD, MDHAR, DHAR, GR, and CAT was proposed to be S-nitrosylated and/or tyrosine nitrated *in vivo* in unstressed *A. thaliana*, salt-stressed citrus (*Citrus aurantium*), GSNO-treated potato or rice injected with H_2O_2 for eliciting cell death (Tanou et al., 2009, 2010; Fares et al., 2011; Kato et al., 2012; Lin et al., 2012). S-nitrosylation, however, was only confirmed for APX from GSNO-treated potato leaves (Kato et al., 2012). In the same study DHAR was demonstrated to be S-nitrosylated and inhibited by NO. A possible target Cys essential for enzymatic function was revealed by point mutation of candidate Cys residues. Human manganese SOD is

a mitochondrial protein that undergoes site-specific nitration at Tyr34 during inflammation. Inactivation of Mn-SOD by nitration provokes oxidative stress and ultimately dysfunction of mitochondria (Radi, 2013). It would be interesting to elucidate if plant SODs are targets of nitrating species with possible roles e.g., in PCD. Collectively, the discussed data suggest that APX, CAT, and DHAR are good candidates for NO-regulated antioxidant enzymes in plants. A systematic approach is needed for deciphering, which antioxidant enzymes are controlled by NO under stress conditions, and what are the underlying molecular mechanisms.

We mentioned before that NO bioactivity has been implicated both in increased as well as decreased antioxidant enzyme activities and ROS levels. One way of explaining the contradictory findings is based on the hypothesis that NO has a dose-dependent effect on the cellular redox status (**Figure 2**) (Thomas et al., 2008). At low concentrations NO might stimulate the antioxidant system and promote cell survival while high concentrations of NO cause severe cell damage and even death. In this model trace NO would preferably react with nucleophiles such as lipids, DNA and metal centered proteins but also with oxygen species forming oxidizing and nitrating species including $ONOO^-$ and NO_2. Little damage and NO-induced signaling will be perceived by the cell triggering antioxidant defence and repair mechanisms. Profound NO production, on the other hand, would promote secondary reactions of NO_2 and $ONOO^-$ with NO and consequently the accumulation of N_2O_3. This would shift conditions in the cell from weak oxidative stress toward heavy nitrosative stress, which—according to the hypothesis of Thomas et al. (2008)—inflicts severe damage ultimately leading to cell death. For some biological effects the duration of NO production is decisive because certain target molecules bind NO very slowly or need sequential NO and ROS modifications (Thomas et al., 2008). Thus, in addition to the chemical environment of the cell, which defines the RNS/ROS composition, the extent of NO production is critical in shaping stress signaling by NO.

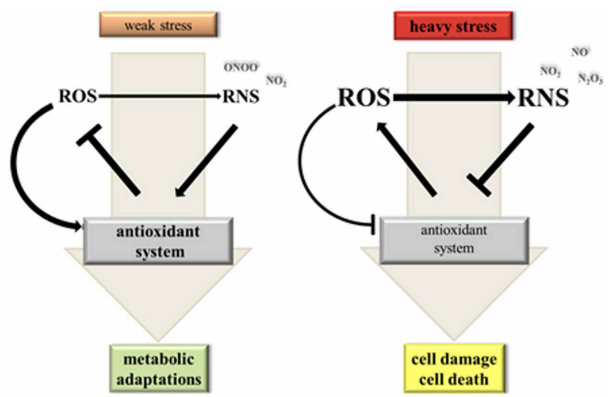

FIGURE 2 | Hypothetical model on the dynamic interaction between NO, ROS and the antioxidant system under stress conditions. Weak stress triggers a moderate elevation of ROS (reactive oxygen species) and NO levels. ROS act as signals inducing NO synthesis and activation of the antioxidant system for improved metabolic adaptation. If ROS is produced at a somewhat higher rate than NO there would be mainly formation of oxidizing and nitrating RNS (reactive nitrogen species) imposing a weak oxidative stress to the cell. Heavy stress leads to a strong ROS and RNS burst. High NO levels promote formation of N_2O_3 from NO_2 and NO and consequently nitrosative stress. Under these conditions ROS and RNS inhibit the antoxidant system causing damage and ultimately death of plant cells.

INTERACTIONS BETWEEN NO AND ANTIOXIDANTS

The versatility of signaling by RNS and ROS is further extended by their interaction with antioxidants. Reduced ascorbate does not react with NO but with nitrosating species NO^+, N_2O_3 and with S-nitrosothiols (Scorza et al., 1997; Kytzia et al., 2006). Consequently, NO is released and AsA is converted to DHA (Combet et al., 2010). DHA spontaneously decays to the ascorbyl radical, which can combine with NO to give O-nitrosoascorbate. The latter finally undergoes hydrolysis to ascorbate and NO_2^- (Kytzia et al., 2006). AsA can also scavenge $ONOO^-$ with rather slow kinetics at neutral pH but rapid kinetics at pH 5.8 yielding NO_2^- and NO_3^- via unknown intermediates (Kurz et al., 2003). Likewise, GSH affects $ONOO^-$ levels either by reduction to NO_2^- or by radical-radical interactions of NO_2 with the glutathiyl radical resulting in the formation of nitroglutathione $GSNO_2$, which in turn can release NO (Balazy et al., 1998). Moreover, GSH effectively prevents $ONOO^-$ mediated tyrosine nitration by re-reducing tyrosyl radicals and catalysing the formation of non-nitrating O_2NOO^- from NO_2 and O_2^- (Kirsch et al., 2001). The biological significance of the above proposed pathways of $ONOO^-$ degradation remains to be investigated. However, the high concentrations of GSH and AsA in plant cells could contribute to maintaining low levels of NO derivatives under non-stress conditions.

Other known plant scavengers of $ONOO^-$ include gamma-tocopherol (vitamin E; Desel et al., 2007), carotenoids and the flavonoids ebselen, epicatechin and quercetin (Haenen et al., 1997). Some of the above compounds are not specific for $ONOO^-$ but scavenge NO and ROS, too. Recently, cytokinins were demonstrated to be involved in controlling NO levels in *A. thaliana* (Liu et al., 2013). Continuous root-uptake of 120 μM SNP severely inhibited growth of *A. thaliana* WT plants whereas the mutant line cnu-1/amp1 was resistant to the same NO treatment. Further characterization of the mutant revealed a correlation between NO resistance and elevated cytokinin levels. Accordingly, WT plants infiltrated with the cytokinin zeatin displayed improved growth on SNP-loaded agar medium. *In vitro*, zeatin was nitrated by peroxynitrite, which produced 8-nitro-zeatin. *In vivo*, SNP caused strong accumulation of 8-nitro-zeatin in cnu-1 as compared to WT. From these results, the authors concluded that cytokinins regulate NO levels by binding the NO derivative $ONOO^-$ (Liu et al., 2013).

NO interacts with glutathione in various ways. At the transcriptional level SNP and GSNO stimulated genes involved in GSH synthesis causing elevated levels of total glutathione in *Medicago truncatula* roots (Innocenti et al., 2007). Accordingly, NO donor treatment triggered an increase in total glutathione in 8 of 10 studies summarized in **Table 1**. In contrast, SNP had no strong effect on GSH concentrations in tobacco BY-2 cells (De Pinto et al., 2002). At the level of chemical interactions GSH binds NO by S-nitrosylation. GSNO is formed either after (1) ROS-induced accumulation of glutathiyl radicals, which bind NO with

rate constants near the diffusion-controlled limit (Madej et al., 2008) or after (2) S-nitrosylation of GSH by nitrogen oxides such as NO^+ and N_2O_3 (Broniowska et al., 2013). GSNO then functions as storage and transport form of NO. It is regarded as an endogenous NO donor, which releases free NO (2 GSNO → 2 NO + GSSG) or S-nitrosylates proteins by transferring the nitroso adduct (Broniowska et al., 2013; Mur et al., 2013).

ENZYMATIC REGULATION OF NO HOMEOSTASIS BY GSNOR, HEMOGLOBIN AND PRO- AS WELL AS ANTIOXIDANT ENZYMES

Levels of the S-nitrosylated tripeptide GSNO are tightly controlled by the enzyme GSNOR. This GSH-dependent formaldehyde dehydrogenase catalyzes the transformation of GSNO to GSSG and hydroxylamine (NH_2NO) in the presence of GSH and NADH as the reducing species (**Figure 3**) (Liu et al., 2001; Sakamoto et al., 2002). In *A. thaliana* silencing or mutation of *GSNOR1* caused accumulation of S-nitrosothiols, NO and NO_3^- indicating that the corresponding enzyme is a major player in NO homeostasis (Sakamoto et al., 2002). *GSNOR1* deficient plants were severely affected in growth and development (Kwon et al., 2012). They also showed increased resistance to the herbicide paraquat and altered responses toward heat stress and pathogen infection (Diaz et al., 2003; Feechan et al., 2005; Rusterucci et al., 2007; Lee et al., 2008; Chen et al., 2009; Holzmeister et al., 2011). In addition to control of NO levels, GSNOR is also indirectly involved in protein denitrosylation because GSNO and S-nitrosylated proteins are in equilibrium (Benhar et al., 2009; Malik et al., 2011). For more information on GSNOR functions refer to recent reviews (Leitner et al., 2009; Gaupels et al., 2011a; Mur et al., 2013). In mammalian/human cells CuZn-SOD and GPX (glutathione peroxidase) were proposed to use GSNO as a substrate and might act in protein denitrosylation without physiological functions being well-established yet (Benhar et al., 2009).

Another upcoming topic is the modulation of NO homeostasis by plant hemoglobins. Class-1 Hb1 catalyse the turnover of NO to NO_3^- thereby influencing growth, development and stress responses (**Figure 3**) (Hill et al., 2010; Hebelstrup et al., 2012). Particularly, the role of alfalfa and *A. thaliana* Hb1 in hypoxia has been studied in more detail (Dordas et al., 2003; Perazzolli et al., 2004; Hebelstrup et al., 2012). It was shown that hypoxia triggered expression of the Hb1-coding gene in roots, probably for confining the stress-induced accumulation of NO. Reduced expression of *Hb1* in transgenic and mutant lines caused an increase in NO levels concomitant with decreased plant growth whereas *Hb1* over-expression improved plant fitness during hypoxia. By scavenging NO the plant might suppress a costly defence response for saving energy and valuable nitrogen under limited oxygen availability (Hebelstrup et al., 2012). Recently, Hb1 was found to be involved in pathogen resistance. *A. thaliana* mutants defective in the Hb1-coding gene *GLB1* were more resistant to the hemibiotrophic *P. syringae* and the necrotrophic fungus *Botrytis cinerea* (Mur et al., 2012). The mutant phenotype was reversed by over-expression of *GLB1* under control of the 35S promoter. The enhanced resistance in the *glb1* mutant correlated with accumulation of SA, JA, and ET. *GLB1* was down-regulated in WT plants during infection, which probably facilitated the induction of defence responses by NO accumulation.

Notably, human hemoglobin degrades $ONOO^-$ to NO_3^- *in vitro* further extending possible functions of hemoglobins in NO signaling (Romero et al., 2003). By comparison plants have evolved efficient mechanisms for enzymatic detoxification of $ONOO^-$ by thiol-dependent peroxidases. The *A. thaliana* peroxiredoxin IIE (PrxII E) and glutathione peroxidase 5 (Gpx5) of poplar both reduce $ONOO^-$ to NO_2^- (**Figure 3**) (Sakamoto et al., 2003; Romero-Puertas et al., 2008; Ferrer-Sueta and Radi, 2009). Both enzymes are then reactivated by thioredoxin in a NADPH-consuming manner. Hence, thioredoxin functions include ROS and $ONOO^-$ scavenging as well as protein denitrosylation illustrating again the essential roles of this enzyme in ROS and RNS control.

At neutral (but not acidic) pH NO_2^- is a rather stable decomposition product of NO and its derivatives. However, a number of plant enzymes can convert NO_2^- to RNS most prominent examples being nitrite reductase and nitrate reductase, which reduce NO_2^- to NO (Stöhr et al., 2001; Morot-Gaudry-Talarmain et al., 2002; Gupta et al., 2011). During severe hypoxia deoxygenated *A. thaliana* Hb1 might act as nitrite reductase although with rather slow kinetics (Tiso et al., 2012). Given the high concentrations of NO_2^- in hypoxic plant tissues Hb1 might still significantly contribute to NO accumulation (Sturms et al., 2011). A more widespread phenomenon could be the nitration-promoting activity of peroxidases. For instance, three *A. thaliana* hemoglobins and Hb1 of *Medicago sativa* were capable of mediating protein nitration via NO_2^- oxidation to NO_2 by a H_2O_2-dependent peroxidase activity (Sakamoto et al., 2004; Maassen and Hennig, 2011). Sakihama et al. (2003) demonstrated the enzymatic nitration of *p*-coumaric acid by action of horseradish peroxidase in the presence of NO_2^- and H_2O_2. All the above data on Hb1 acting as nitrite reductase and enzymatic nitration by peroxidases were obtained *in vitro* and it is difficult to draw any meaningful conclusions for the *in vivo* situation.

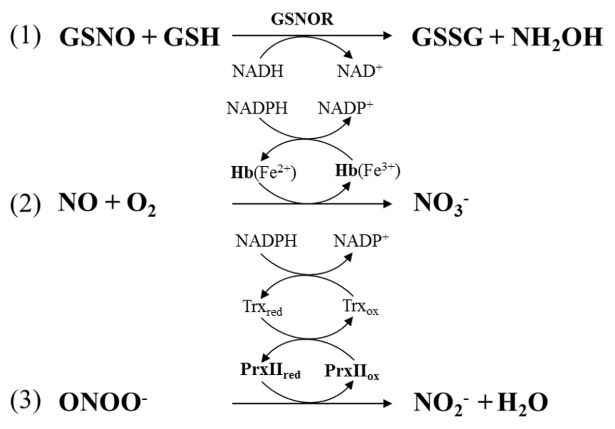

FIGURE 3 | Enzymatic regulation of NO homeostasis by (1) S-nitrosogutathione reductase (GSNOR), (2) hemoglobin (Hb), and (3) peroxiredoxin IIE (PrxIIE). PrxIIE is reduced by thioredoxin (Trx).

NO AND REDOX SIGNALING IN CELL DEATH

ROS and RNS are major players in plant stress signaling. In this section we will survey current knowledge on the roles of ROS, RNS and elements of the antioxidant system in cell death events induced by biotic and abiotic stressors. Plant PCD was described as a genetically controlled cell suicide exhibiting marked similarities but also considerable differences to apoptosis in animal/human cells (Mur et al., 2008; De Pinto et al., 2012). Plants attacked by an avirulent pathogen develop HR, which is a defence mechanism for restricting the spread of pathogens by cell wall reinforcement, production of defensive secondary metabolites and ultimately cell death (Mur et al., 2008).

Almost 20 years ago Chris Lamb and his co-workers discovered that soybean cells infected with avirulent *Pseudomonas syringae pv. glycinea* accumulated high levels of H_2O_2, which functioned as a cell death inducer during the HR (Levine et al., 1994). Suppression of the pathogen-induced H_2O_2 burst by the NADPH oxidase inhibitor diphenylene iodonium (DPI) prevented cell death whereas low millimolar concentrations of exogenous H_2O_2 triggered HR-PCD in a calcium-dependent manner (Levine et al., 1994, 1996). Later, researchers of the same group demonstrated that NO was another essential messenger in cell death execution (Delledonne et al., 1998). Application of a NO scavenger and a NOS activity inhibitor both reduced HR-PCD of soybean suspension cells infected with avirulent bacterial pathogens. Importantly, SNP triggered cell death most efficiently in conjunction with ROS but not in the presence of DPI or CAT. ROS donors in turn efficiently killed soybean cells only if applied together with SNP (Delledonne et al., 1998). Comparable results were obtained with tobacco BY-2 cells. Simultaneous application of SNP and the H_2O_2-generating donor system glucose/glucose oxidase but not each individual donor alone caused a drop in ascorbate and glutathione levels, inhibition of APX and consequently PCD of tobacco BY-2 cells (De Pinto et al., 2002). Therefore, it was postulated that NO and ROS cooperate in cell death signaling (**Figure 2**).

Recent studies have begun to unravel the underlying modes of interactions between NO, ROS and the antioxidant system during PCD. It was shown that $ONOO^-$ arose in *A. thaliana* plants challenged by avirulent *Pseudomonas syringae* (Gaupels et al., 2011b). The peak of $ONOO^-$ formation from NO and O_2^- coincided with the onset of the PCD. In unstressed plants $ONOO^-$ was continuously scavenged by PrxIIE, which was inhibited by S-nitrosylation in course of the HR (Romero-Puertas et al., 2007). The fact that $ONOO^-$ levels are controlled in a sophisticated manner would imply an important role of this RNS in the induction of cell death and pathogen resistance. However, contrary to mammalian cells this RNS does not kill plant cells (Delledonne et al., 2001). It was demonstrated that SOD, GR, CAT, and APX, which are all involved in ROS depletion, can be tyrosine nitrated by $ONOO^-$ (Chaki et al., 2009; Lozano-Juste et al., 2011). If this is a significant process *in vivo* remains to be proven.

H_2O_2 rather than O_2^- was proposed to be a pivotal signal in regulating PCD. This particular ROS acts as an inducer of NO synthesis in tobacco cells (De Pinto et al., 2006) and in mutant plants with disturbed redox homeostasis. For instance, rice knock-out mutants defective in a CAT-coding gene showed increased H_2O_2 levels, nitrate reductase-dependent accumulation of NO and spontaneous leaf cell death (Lin et al., 2012). Application of the NO scavenger PTIO mitigated the cell death phenotype. The importance of a down-regulation of ROS detoxifying enzymes during PCD was further corroborated by the finding that overexpression of thylakoidal APX led to a higher resistance against SNP induced cell death (Murgia et al., 2004b). In *A. thaliana* WT plants 5mM SNP triggered H_2O_2 accumulation and cell death, which was both reduced in the transgenic line probably because H_2O_2 was degraded by the elevated APX activity in these plants. The antioxidant enzymes CAT and APX control H_2O_2 levels under mild stress conditions. Severe cadmium stress triggered NO as well as H_2O_2 accumulation and senescence-like PCD of *A. thaliana* suspension cultured cells (De Michele et al., 2009). However, co-treatment with the NOS inhibitor L-NMMA prevented the NO-dependent inhibition of CAT and APX, which in turn reduced H_2O_2 levels and increased cell viability under cadmium stress.

Mechanical wounding provokes cell damage, which could serve as a point of entry into the plant e.g., for pathogenic bacteria. To avoid this, PCD is triggered in intact cells nearby the damaged cells for sealing the wound site. In wounded leaves of *Pelargonium peltatum* NO accumulation was restricted to the site of injury (Arasimowicz et al., 2009). Treatment with cPTIO confirmed that NO inhibited APX and CAT activity thereby temporarily enhancing the H_2O_2 content at the edge of the wound. Pre-treatment of leaves with NO donors before wounding prevented the H_2O_2 burst and reduced necrotic cell death in sweet potato (Lin et al., 2011). The exact mechanism of NO action was not determined but available data suggest that APX, GR, MDHAR and thioredoxin are S-nitrosylated during PCD, which could affect their activity (Murgia et al., 2004b; Lin et al., 2012). Inhibition of GR and MDHAR would also impact on the redox status of the glutathione and ascorbate pools. It should be considered that enzymatic activity can also be influenced by ROS-dependent modifications, which was proposed for oxidation-triggered inhibition of APX (**Figure 2**) (De Pinto et al., 2006). The latter enzyme was also suppressed in gene expression during PCD (De Pinto et al., 2006).

The role of NO in incompatible interactions between *A. thaliana* and avirulent *Pseudomonas syringae* was investigated using transgenic plant lines expressing a bacterial NO dioxygenase (NOD, flavohemoglobin) (Zeier et al., 2004). NOD expression attenuated the pathogen-induced NO accumulation. As a consequence the H_2O_2 burst was diminished and transgenic plants developed less HR-PCD and were delayed in SA-dependent *PR1* expression. These results support again the hypothesis that high levels of NO amplify redox signaling during PCD by inhibiting the plant antioxidant machinery (Zeier et al., 2004). NO and H_2O_2 might mutually enhance each other's accumulation by positive feed-back regulation. To this end, NO and ROS producing enzymes as well as elements of the antioxidant system must be regulated in a highly coordinate fashion for initiation of PCD. The exact signaling pathways remain to be deciphered in future studies.

However, the plant must also constrain stress signaling by NO, ROS and the antioxidant system for avoiding excessive damage by

runaway cell death. Therefore, it is worth mentioning that both ROS as well as NO were found to induce genes involved in cell protection such as a gene coding for glutathione S-transferase (Levine et al., 1994). Yun and colleagues (Yun et al., 2011) even demonstrated inhibition of the ROS-producing enzyme AtRBOHD by NO in *A. thaliana* challenged by avirulent bacteria. The authors proposed a model, in which the early burst of ROS and NO initiates HR-PCD but at later stages of the defence response the SNO levels exceed a certain threshold and subsequently the AtRBOHD is inactivated by S-nitrosylation at Cys 890, which terminates the HR. In contrast to R gene-mediated resistance against avirulent pathogens, bacterial lipopolysaccharides (LPS) elicit basal pathogen resistance without onset of HR-PCD. LPS-induced NO synthesis by an arginine-dependent enzymatic source even protected plant cells against oxidative stress and cell death by enhancing the activities of CAT, SOD, and POD. The changed cellular redox status contributed to the regulation of NPR1-dependent expression of defence genes (Sun et al., 2012). In sum, NO can either act as an inducer or suppressor of plant PCD dependent on its local cellular levels and its tightly controlled interaction with ROS and elements of the antioxidant system (**Figure 2**).

CONCLUDING REMARKS

ROS and NO are increasingly recognized signaling molecules in plant physiology. While research on ROS has a long history NO came into focus only 15 years ago. In the present paper we reviewed recent literature dealing with the interaction between ROS, NO and the antioxidant system during stress defence. As one interesting outcome we found that exposure of plants to unfavorable conditions inevitably induced ROS but not necessarily NO accumulation. ROS can arise as a toxic by-product of disturbed energy metabolism and/or can be produced for signaling purposes. In contrast, NO is rather a highly specialized second messenger, which modifies ROS signaling or acts independently of ROS. Significantly, ROS and NO bursts are often triggered simultaneously—sometimes even in the same cellular compartment. Particularly chloroplasts and peroxisomes are hotspots of NO-ROS interactions. NO, ROS and antioxidants chemically react resulting in the formation of RNS such as $ONOO^-$, NO_2, N_2O_3, and GSNO. More indirect interactions include induction of NO synthesis by H_2O_2 and accumulation of ROS due to inhibition of antioxidant enzymes by NO-dependent protein modifications. Uncontrolled self-amplification of ROS/RNS signaling might provoke nitrosative stress and ultimately PCD. Therefore, plants have developed efficient measures for controlling NO levels by GSNOR, hemoglobins and other RNS scavenging enzymes. This review was also aimed at investigating the extreme versatility of possible reactions between NO, ROS and the antioxidant system. Many of the discussed findings originate from *in vitro* systems or animal/human models. More basic research is urgently needed for defining chemical reactions and their products actually occurring *in planta*.

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Nitric oxide in guard cells as an important secondary messenger during stomatal closure

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The modulation of guard cell function is the basis of stomatal closure, essential for optimizing water use and CO_2 uptake by leaves. Nitric oxide (NO) in guard cells plays a very important role as a secondary messenger during stomatal closure induced by effectors, including hormones. For example, exposure to abscisic acid (ABA) triggers a marked increase in NO of guard cells, well before stomatal closure. In guard cells of multiple species, like *Arabidopsis*, *Vicia* and pea, exposure to ABA or methyl jasmonate or even microbial elicitors (e.g., chitosan) induces production of NO as well as reactive oxygen species (ROS). The role of NO in stomatal closure has been confirmed by using NO donors (e.g., SNP) and NO scavengers (like cPTIO) and inhibitors of NOS (L-NAME) or NR (tungstate). Two enzymes: a L-NAME-sensitive, nitric oxide synthase (NOS)-like enzyme and a tungstate-sensitive nitrate reductase (NR), can mediate ABA-induced NO rise in guard cells. However, the existence of true NOS in plant tissues and its role in guard cell NO-production are still a matter of intense debate. Guard cell signal transduction leading to stomatal closure involves the participation of several components, besides NO, such as cytosolic pH, ROS, free Ca^{2+}, and phospholipids. Use of fluorescent dyes has revealed that the rise in NO of guard cells occurs after the increase in cytoplasmic pH and ROS. The rise in NO causes an elevation in cytosolic free Ca^{2+} and promotes the efflux of cations as well as anions from guard cells. Stomatal guard cells have become a model system to study the signaling cascade mechanisms in plants, particularly with NO as a dominant component. The interrelationships and interactions of NO with cytosolic pH, ROS, and free Ca^{2+} are quite complex and need further detailed examination. While assessing critically the available literature, the present review projects possible areas of further work related to NO-action in stomatal guard cells.

Keywords: abscisic acid, cytosolic pH, elicitors, polyamines, phospholipids, reactive oxygen species, signal transduction

INTRODUCTION

Stomatal pores are the gateways for not only transpirational H_2O loss but also entry of CO_2 into leaves. Due to such dual role, the regulation of stomatal aperture, and yet maintenance of opening are essential to keep up the water balance and at the same time make CO_2 available for photosynthesis. Stomatal opening and closure are mediated by the changes in turgor pressure of guard cells. Stomata open when guard cells are turgid and close when the guard cells are flaccid. As closed stomata restrict pathogen entry into leaves, stomata become key players also in defense response against several pathogens (Underwood et al., 2007; Melotto et al., 2008). Several factors modulate stomatal function, such as drought, light, high CO_2, humidity, and plant hormones, such as ABA (all abbreviations listed on first page). Some of the plant hormones (ABA, MJ, ethylene), salicylic acid, polyamines and even elicitors (mostly microbial) cause stomatal closure, while auxins and cytokinins promote stomatal opening (Bright et al., 2006; Acharya and Assmann, 2009; Alcázar et al., 2010; Jing et al., 2012; Ye et al., 2013).

NO has multifunctional roles in plants: stomatal movement, host-pathogen interactions, hormonal signaling during growth/development and adaptation to abiotic/biotic stress (Delledonne et al., 1998; Bright et al., 2006; Yan et al., 2007; Neill et al., 2008; Wilson et al., 2008, 2009; Siddiqui et al., 2011). In plants, NO can be a signal to induce secondary metabolite accumulation (Lu et al., 2011) and to promote cell death

Abbreviations: ABA, abscisic acid; ABI1/2, ABA-insensitive protein phosphfatase 2C type 1/2; cPTIO, 2-phenyl-4,4,5,5-tetramethyl imidazoline-1-oxyl 3-oxide; cADPR, cyclic ADP ribose; CDPK, calcium-dependent protein kinase; CO, carbon monoxide; CO_2, carbon dioxide; cGMP, cyclic guanosine monophosphate; DAO, diamine oxidase; DGK, diacylglycerol kinase; DAF-2DA, 4,5-diaminofluorescein diacetate; DAG, diacylglycerol; ExtCaM, extra cellular calmodulin; flg22, flagellin 22; GSNO, S-nitrosoglutathione; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; H_2S, hydrogen sulfide; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; MJ, methyl jasmonate; L-NNA, N_ω-nitro-L-arginine; L-NAME, *N*-nitro-L-arginine methyl ester; NR, nitrate reductase; NADPH, Nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; NIR, nitrite reductase; NOA, nitric oxide-associated; LPS, lipopolysaccharide; PAO, polyamine oxidase; PAMP, pathogen-associated molecular pattern; PIP_2, phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; PLD, phospholipase D; PLC, phospholipase C; PP2C, type 2C protein phosphatase; ROS, reactive oxygen species; SA, salicylic acid; SNP, sodium nitroprusside; XOR, xanthine oxidoreductase; YEL, yeast elicitor; PYR/PYL/RCAR, pyrabactin resistance protein1/PYR-like proteins/regulatory components of ABA receptor.

(Gupta et al., 2011b; Bellin et al., 2013). The production of NO in stomatal guard cells has been known since several years (Desikan et al., 2002; Garcia-Mata et al., 2003). But the mechanisms of NO action and interaction with other signaling components in guard cells have been studied in detail, since only a few years. The rise in NO of guard cells is a common and dominant event during stomatal closure induced by several effectors and in different plants (**Table 1**).

Table 1 | The rise in NO of guard cells as a common event during stomatal closure induced by hormones, elicitors or environmental factors.

Effector	Source *in vivo*	Test plant	References
PLANT HORMONES			
ABA	Endogenous	*Vicia faba* *Pisum sativum* *Arabidopsis thaliana*	García-Mata and Lamattina, 2002 Gonugunta et al., 2008 Neill et al., 2008; Islam et al., 2010
MJ	Endogenous	*A. thaliana* *V. faba*	Munemasa et al., 2007; Saito et al., 2009 Xin et al., 2005
SA	Endogenous	*V. faba, Commelina communis* *A. thaliana* *Lycopersicon esculentum*	Xin et al., 2003 Sun et al., 2010; Khokon et al., 2011 Poór and Tari, 2012
Ethylene	Endogenous	*A. thaliana* *V. faba*	Jing et al., 2010 Liu et al., 2012
BIOTIC STRESS COMPONENTS (ELICITORS)			
Chitosan	Derivative of chitin fragments from fungal cell wall	*L. esculentum, C. communis* *P. sativum* *A. thaliana*	Lee et al., 1999 Srivastava et al., 2009 Khokon et al., 2010b
Flg22*	22 amino acid peptide from Flagellin, bacterial flagellar protein	*A. thaliana*	Melotto et al., 2006
LPS*	Glycolipid component of gram negative bacterial outer membrane	*A. thaliana*	Melotto et al., 2006
E. coli O157:H7	Human pathogen	*A. thaliana*	Melotto et al., 2006
Harpin	*Xanthomonas oryzae*	*Nicotiana benthamiana*	Zhang et al., 2009a, 2012b
INF1	*Phytophthora infestans*	*N. benthamiana*	Zhang et al., 2009a
Boehmerin	*Phytophthora boehmeriae*	*N. benthamiana*	Zhang et al., 2009a, 2012b
Nep1	*Magnaporthe oryzae*	*N. benthamiana*	Zhang et al., 2012b
YEL (Yeast elicitor)	Yeast extract	*A. thaliana*	Khokon et al., 2010a
Oligochitosan	Fragment of chitosan prepared by enzymatic hydrolysis	*Brassica napus*	Li et al., 2009b
ENVIRONMENTAL FACTORS			
UV-B	Environment	*V. faba* *A. thaliana*	He et al., 2005 He et al., 2013
Bicarbonate (mimics high CO_2)	Environment	*P. sativum*	Kolla and Raghavendra, 2007
SIGNALING COMPONENTS			
$CaCl_2$ (Buffered)	Endogenous	*A. thaliana*	Wang et al., 2012
H_2O_2	Endogenous	*V. faba* *A. thaliana*	He et al., 2005 Bright et al., 2006
Calmodulin	Endogenous	*A. thaliana*	Li et al., 2009a

**PAMP- the term used for elicitors like flg22, LPS.*

There has been growing interest in NO as an essential signal molecule during stomatal closure, and plant growth/development, besides defense against pathogens. The ABA-induced stomatal closure is associated with a rise in NO as well as ROS of guard cells. The rise in NO causes elevation of free Ca^{2+} in guard cells, restriction of K^+ influx and promotion of anion efflux (Garcia-Mata et al., 2003; Sokolovski and Blatt, 2004), all resulting in loss of guard cell turgor and stomatal closure. This article emphasizes that NO is a common factor during stomatal closure induced by varying factors, including hormones, microbial elicitors (yeast/bacterial/fungal/pathogen) and abiotic environmental stresses. The possible sources of NO are described, highlighting the ambiguity on the role of true NOS in plants. A pathway of signal transduction, with the components involved in NO action, is proposed. Attention is drawn toward the interaction of NO with other signaling components in guard cells. Finally, a few of the emerging topics and unresolved questions, for further research are indicated.

In view of the large number of reports on the rise in NO of guard cells in relation to stomatal closure, we had to limit references to original articles, published in the last 5 years. There are excellent reviews covering the earlier work on the role of NO during stomatal closure (García-Mata and Lamattina, 2002, 2013; Neill et al., 2003, 2008; Desikan et al., 2004; Lamotte et al., 2005; Wilson et al., 2008, 2009; Hancock et al., 2011) and the importance of NO during the innate immunity responses of plants (Wendehenne et al., 2004; Leitner et al., 2009; Gaupels et al., 2011; Yoshioka et al., 2011). The importance of NO as a general signaling molecule in several processes of growth and development have been reviewed elsewhere (Durner and Klessig, 1999; Lamattina et al., 2003; Moreau et al., 2010; Baudouin, 2011; Fröhlich and Durner, 2011; Martínez-Ruiz et al., 2011; Astier et al., 2012; Simontacchi et al., 2013).

HORMONES: ABA, ETHYLENE, METHYL JASMONATE

The rise in NO is a common step during stomatal closure induced by hormones like ABA; or elicitors like chitosan; and even abiotic stress conditions (**Table 1**). Among the plant hormones, the perception and action of ABA is well characterized (Sirichandra et al., 2009; Cutler et al., 2010; Raghavendra et al., 2010). The stomatal closure induced by ABA involves a series of events, including a rise in reactive nitrogen species i.e., nitric oxide (NO). Additional signaling components that are involved are: reactive oxygen species (ROS, mostly H_2O_2), cytosolic Ca^{2+}, cytoplasmic pH, G-proteins, protein kinases as CDPK and MAPK, protein phosphatases, phospholipases and sphingolipids (Gonugunta et al., 2008; Neill et al., 2008; Wang and Song, 2008; Umezawa et al., 2010; García-Mata and Lamattina, 2013). Extensive studies on guard cells of *Arabidopsis*, pea, *Vicia faba* and *Commelina communis* have established that NO is an essential signaling component during ABA-induced stomatal closure (Xin et al., 2005; Gonugunta et al., 2008, 2009; Neill et al., 2008). The increase in NO is usually associated with the elevated ROS levels, particularly H_2O_2, generated by plasma membrane NADPH oxidase. The role of several signaling components involved in NO production and stomatal closure induced by ABA was convincingly demonstrated by studies performed in Arabidopsis mutants (**Table 2**). The impaired NO production by ABA in *nia1,nia2* mutants (Desikan et al., 2006) and in *atrbohD/F* mutant is an indication of the key roles of NR and NADPH oxidase, respectively (Bright et al., 2006).

The other hormones, which induce an increase in NO leading to stomatal closure, are ethylene and MJ. External application of ethephon (an ethylene-releasing compound) or 1-aminocyclopropane-1-carboxylic acid (the precursor of ethylene) induced stomatal closure in a dose-dependent manner in *Arabidopsis thaliana* (Desikan et al., 2006). Ethylene-induced stomatal closure was associated with a rise in not only NO, but also H_2O_2, Ca^{2+}, and cytoplasmic pH (Jing et al., 2010, 2012). The precise order of these molecules during NO action and stomatal closure is not yet known. The effects of ethylene on NO level may be either direct or indirect through the modulation of endogenous ABA levels. This aspect needs additional experiments for confirmation.

MJ, a linolenic acid derivative, is as powerful as ABA in inducing stomatal closure, and elevating the levels of NO, besides ROS in guard cells (Gonugunta et al., 2009; Munemasa et al., 2011b). The role of NO as one of the signaling components during MJ-induced stomatal closure is further confirmed by the decrease in NO production and stomatal closure by L-NAME in *V. faba* guard cells (Xin et al., 2005). The MJ or ABA-induced NO production was impaired in *rcn1* mutant of *A. thaliana*, deficient in the regulatory subunit of protein phosphatase 2A (RCN1) (Saito et al., 2008, 2009). However, SNP (a NO donor) induced stomatal closure along with rise in guard cell NO levels in *rcn1* mutant as well as in wild type.

MICROBIAL ELICITORS

Besides being gateways for water/CO_2, stomata can limit the invasion of pathogenic bacteria, and thus be a part of the plant innate immune system (Baker et al., 2010; Zeng et al., 2010). A burst in NO production has long been identified as one of the plant defense responses. Further, NO plays a very important role in cell death and activation of defense genes against plant pathogens (Delledonne et al., 2003; Romero-Puertas et al., 2004; Garcia-Brugger et al., 2006). The protective role of NO doubles up, as it upregulates secondary metabolism, and levels of antimicrobial compounds (Wang and Wu, 2004; Zhang et al., 2012a). In view of such crucial role, the molecular events in plant cells, triggered by NO, to help in innate immunity have been studied in detail. Compared to the extensive literature on the role of the NO-burst as a component of pathogen resistance, there is very limited work on the mechanism of NO-rise in guard cells, when exposed to elicitors/plant pathogens.

A typical effect of several elicitors is the marked stomatal closure and an increase in guard cell NO (**Table 1**). NO production was observed in guard cells of *A. thaliana*, *Pisum sativum,* and *Nicotiana benthamiana* in response to elicitors such as, PAMP, chitosan and oligochitosan (Melotto et al., 2006; Li et al., 2009b; Srivastava et al., 2009). In addition, other elicitors such as harpin, boehmerin, INF1, and Nep1 induced the production of NO in guard cells of *N. benthamiana* (Zhang et al., 2009a, 2012b). Impaired stomatal closure in response to elicitors by cPTIO (NO scavenger) or upon treatment with L-NNA (NOS inhibitor)

Table 2 | Use of Arabidopsis mutants to demonstrate the importance of signaling components involved in the rise of NO during stomatal closure.

Mutant	Deficiency in mutant	Effector used for NO rise	Impairment in the plant	References
abi1-1 and *abi2-1*	Protein phosphatase 2C	ABA	Stomatal closure but not NO production	Desikan et al., 2002
aba2-2	Protein phosphatase 2C	Methyl jasmonate	NO and ROS production	Ye et al., 2013
atrbohD/F	NADPH Oxidase	ABA	H_2O_2 production	Bright et al., 2006
coi1 and *abi2-1*	Coronatine-insensitive1 protein (COI1) and protein phosphatase 2C	Methyl jasmonate	ROS and NO production	Munemasa et al., 2007
cpk6-1	Calcium dependent protein kinase	ABA and MJ	NO levels; no change in ROS	Munemasa et al., 2011a
gpa1-1, gpa1-2 atnoa1 and *atrbohD/F*	G-protein α sub unit and NADPH Oxidase	Extracellular calmodulin (ExtCaM)	NO rise in guard cell and stomatal closure	Li et al., 2009a
nia1 and *nia2*	Nitrate reductase	Salicylic acid and ABA	NO rise in guard cell and stomatal closure	Bright et al., 2006; Hao et al., 2010
pldα1	Phospholipase Dα1	ABA	NO production	Zhang et al., 2009b
Pldδ-1/pldα1	Phosholipase Dα and Dδ	ABA	NO production only, but not stomatal closure	Distéfano et al., 2012
rcn1	Regulatory subunit of protein phosphatase 2A	Methyl jasmonate	NO production	Saito et al., 2009

confirms the role of NO in stomatal signaling (Melotto et al., 2006; Khokon et al., 2010a,b; Zhang et al., 2012b).

The production of NO occurred downstream of ROS, during stomatal closure induced by chitosan (Srivastava et al., 2009; Khokon et al., 2010b). The signaling components identified with elicitor-induced stomatal closure and NO-rise in guard cells are: ROS/NADPH oxidases, G-proteins, vacuolar processing enzyme (Zhang et al., 2009a, 2010, 2012b). It is not clear if the signal transduction chain involving NO-rise and stomatal closure induced by different elicitors follows the same or a modified pathway.

SALICYLIC ACID

SA is a phenolic compound, known to play a key role in a wide range of physiological and developmental processes, such as thermogenesis, fruit ripening, ethylene synthesis and plant defense against pathogens (Loake and Grant, 2007). There have been early reports on the regulation by SA of stomatal movement (Manthe et al., 1992; Lee and Joon-Sang, 1998) and role of signaling molecules, such as superoxide radicals, Ca^{2+}, H_2O_2, and NO in modulating SA-effects (Mori et al., 2001). The SA-induced NO production and stomatal closure was impaired by cPTIO (NO scavenger) in guard cells of *V. faba* (Xin et al., 2003) and Arabidopsis (Khokon et al., 2011) highlighting the importance of NO during responses to SA.

PHOSPHOLIPIDS

Phospholipids are major components of plasma membrane and have emerged as key signaling molecules (Meijer and Munnik, 2003; Testerink and Munnik, 2005; Wang, 2005). These phospholipids such as phosphatidic acid (PA), phosphatidylinositol 4,5-bisphosphate (PIP_2) and diacylglycerol (DAG) regulate a wide range of growth and developmental processes including ABA signaling, programmed cell death and defense response (Katagiri et al., 2005; Wang, 2005; Choi et al., 2008). Another group of phospholipids, which could potentially interact with NO, are sphingolipids (Guillas et al., 2013). The role of sphingolipids in relation to NO-action on guard cells needs to be probed in detail.

Among the phospholipids, the effect of PA appears to be quite interesting. In plant tissues, PA generated by either PLC or PLD, can inactivate K^+_{in} channels and promote stomatal closure (Jacob et al., 1999; Uraji et al., 2012). The increase in the levels of PA in *V. faba* guard cells on exposure to NO and prevention of stomatal closure by inhibitors of either PLC or PLD suggested that NO might be involved in the production of PA and stomatal closure (Distéfano et al., 2008). Among the 12 PLD genes of Arabidopsis, PLDα and PLDδ were shown to be involved in stomatal regulation (Zhang et al., 2009b; Distéfano et al., 2012; Uraji et al., 2012). Further description is in the section on "Signaling components in guard cells during NO action."

POLYAMINES

Polyamines are ubiquitous, low molecular weight nitrogenous aliphatic compounds, which regulate several physiological and developmental functions (Kusano et al., 2008). Although the exact mechanisms are not completely understood, polyamines seem to help in plant adaptation to both biotic and abiotic stress (Alcázar et al., 2010). There are indications that polyamines interact with ABA (Alcázar et al., 2006, 2010). The limited reports on the increase in NO production by polyamines are ambiguous. Flores et al. (2008) observed that upregulation of arginase activity reduced the release of NO in *A. thaliana* mutants. In contrast, polyamines elevated NO production in tobacco BY-2 cells and *Ocotea catharinensis* somatic embryo cultures (Santa-Catarina et al., 2007). Among the three polyamines tested, spermine was the most effective in inducing NO production, followed by spermidine and putrescine. Arginine, despite being a precursor molecule for the polyamine biosynthesis, could not increase NO (Tun et al., 2006).

The increase in NO of guard cells by polyamines may be related to H_2O_2. Oxidation of putrescine by DAO can facilitate ABA-induced H_2O_2 production (An et al., 2008). When polyamines are catabolized by DAO or PAO, H_2O_2 is produced as one of the products (Alcázar et al., 2010). Though speculative, it appears reasonable to expect that the polyamine catabolic byproduct of H_2O_2 can elevate NO, as NO acts downstream of relation to H_2O_2 during stomatal closure (Srivastava et al., 2009). Further studies are required to clarify if polyamines have a direct or indirect effect on the production of NO and ROS in stomatal guard cells.

SOURCES OF NO

The levels of NO within the cell, depends on the balance between production and scavenging. There is considerable work on the sources of NO in plant tissues, but very little information is available on the modes of scavenging NO. The possible sources of NO production can be categorized as enzymatic or non-enzymatic. Gupta et al. (2011a) summarized the literature on the sources of NO in plants, proposing that seven possible routes of NO production can be identified. In plants, the NR mediated NO production is accepted widely, while there is ambiguity about the role of a true NOS. Neill et al. (2008) reported that ABA-induced NO synthesis in guard cells could be driven by both NOS-like enzyme and NR activity. Nitrate can be reduced to nitrite and then to NO by NR, using NADP(H) as an electron source (Besson-Bard et al., 2008; Baudouin, 2011). However, the capability of NR in NO production is calculated to be only about 1% of its nitrate reduction capacity (Planchet et al., 2005). The root specific Ni-NOR found in purified plasma membranes of tobacco (*Nicotiana tabacum*) roots, has been proposed to be involved in the reduction of apoplastic nitrite to NO (Stöhr and Stremlau, 2006). The role of such plasma membrane bound nitrite: NO reductase (Ni-NOR) in guard cell NO production is yet to be critically assessed.

The NOS-induced NO production is well documented in animal systems, with reports of three isoforms: inducible, neuronal and endothelial NOS (Alderton et al., 2001). However, the existence of true NOS in plants is strongly questioned, because of two major reasons: (i) apparent absence of NOS in the genome of plants, including Arabidopsis; (ii) no convincing evidence for a protein, with NOS-like activity in higher plants. Although proteins with supposedly NOS activity are occasionally reported (Fröhlich and Durner, 2011), their exact identity is questionable. One of the NOS-like enzymes, described earlier (Moreau et al., 2010), turned out to be a GTPase and renamed as NOA. The role of NOA in NO production appears to be a possibility. Despite intense efforts, a true NOS is yet to be discovered in higher plants. The nearest finding is the report on arginine-dependent NOS-like activity in a green alga, *Ostreococcus tauri* (Foresi et al., 2010). The ambiguity on the source of NO extends to SA-mediated NO-production, with reports implicating the importance of NOS-like enzyme (Xin et al., 2003; Sun et al., 2010) or NR (Zottini et al., 2007; Hao et al., 2010). Immediate attention is required to identify the precise enzymatic source of NO production in guard cells, and such information would be applicable to other plant tissues.

There is an additional possibility of NO production by non-enzymatic reactions. Two such instances are: (i) Reduction of nitrite to NO occurred under the acidic and highly reduced conditions, and such NO formation was not impaired by typical NOS inhibitors (Zweier et al., 1999); and (ii) *Rapid* production of NO from nitrite in the incubation medium, *Hordeum vulgare* (barley) aleurone layers further promoted by phenolic compounds (Bethke et al., 2004). However, the relevance of these non-enzymatic NO sources in guard cells are unclear, and these may not be as crucial as enzymatic ones.

Our current knowledge of biological scavenging mechanisms of NO in plants, is quite meagre. Being diffusible, NO can react with several molecules within the cell. Such decrease in NO, due to its highly reactive nature should be considered important. There are reports that GSH and plant hemoglobins, could scavenge NO (Perazzolli et al., 2004; Basu et al., 2010), but the exact enzymatic steps of NO conversion need to be elucidated. The nitrosylation of cellular proteins could be involved in the NO action as well as the maintenance of NO levels. For example, nitrosylation has been found to affect the activity of proteins, such as GAPDH (Lindermayr et al., 2005; Vescovi et al., 2013; Zaffagnini et al., 2013) and outward K^+-rectifying channels (Sokolovski and Blatt, 2004).

SIGNALING COMPONENTS IN GUARD CELLS DURING NO ACTION

Several signaling components have been identified to act either upstream or downstream of NO. The role of different components was established by usually three sets of evidence: (i) Employing inhibitors or scavengers, (ii) Monitoring the components by suitable fluorescent dyes; and finally (iii) Validation by using mutants deficient in a given component of signal transduction chain (**Table 2**). The inhibitors related to NO are: cPTIO (scavenger of NO), L-NAME (inhibitor of NOS) and tungstate (inhibitor of NR). In some studies, artificial NO donors such as SNP and GSNO are also used. Studies on real-time monitoring of NO production, during stomatal closure have demonstrated that pH and ROS of guard cells rise before that of NO and stomatal closure occurs subsequently. Such early rise in pH and ROS was observed during stomatal closure induced by ABA, MJ as well as chitosan (Suhita et al., 2004; Gonugunta et al., 2008, 2009; Srivastava et al., 2009). Studies using NO scavenger

(cPTIO) or L-NAME and tungstate, inhibitors of "NOS-like" and NR prevented the NO production but not ROS during stomatal closure in epidermal strips. Among the signaling components: PYR/PYL/RCAR (ABA-receptor proteins), ABI1/2 (that help binding to receptor proteins), ROS (generated by NADPH oxidase), pH, G-proteins and PA/PLC/PLDα1 act upstream of NO rise (Sirichandra et al., 2009; Zhang et al., 2009b; Cutler et al., 2010). In contrast to the role of PLDα1, PLDδ is reported at either upstream or downstream of NO production in guard cells (Distéfano et al., 2012; Uraji et al., 2012). Similarly, Ca^{2+} may act at both levels upstream and downstream of NO (Garcia-Mata et al., 2003; Gonugunta et al., 2008).

Unlike other reports, an intriguing observation was that ABI1 and ABI2 might act downstream of the NO in stomatal signaling by ABA in *A. thaliana* guard cells (Desikan et al., 2002). Studies with mutants deficient in ROS production (like *rbohD/F*) and by inhibitors like DPI, confirmed the strong association between ROS and NO (Bright et al., 2006; Neill et al., 2008; Srivastava et al., 2009). The stomatal closure induced by ABA or H_2O_2 and associated NO production were impaired in *nia1,nia2* double mutant (Bright et al., 2006). The NO production by microbial elicitors (boehmerin, harpin and INF1) was impaired in *NbrbohA* and *NbrbohB* single and double silenced plants confirming that ROS acted upstream of NO production (Zhang et al., 2009a). Similarly, limited stomatal closure and NO production in response to microbial elicitors (harpin, Nep1, boehmerin) in G-protein (*G*α-, *G*β1-, and *G*β2-) silenced plants of *N. benthamiana* prove that G-proteins facilitate NO production, before stomatal closure (Li et al., 2009a; Zhang et al., 2012b).

The ability of PA to interact with ABI1 and NADPH oxidase (Zhang et al., 2004) implies that PA may act either upstream or downstream of NO. Distéfano et al. (2008, 2010) have established that the rise in NO causes elevation of PA which acts downstream of the NO during stomatal closure in *V. faba*. In the signaling scheme, proposed by Distéfano et al. (2010), ABA-induced NO activates PLC and/or PLD pathways to generate PA (Zhang et al., 2009b; Uraji et al., 2012). One of the products of PLC, namely IP_3 can induce the release of Ca^{2+} from internal stores leading to stomatal closure. Attention needs to be drawn to reported participation of the PI3 and PI4 kinases (Kolla and Raghavendra, 2007) in bicarbonate-induced NO production. Such pathway is extremely interesting and may represent ROS-independent route of NO-production.

A direct well-known effect of NO is it's up-regulation of Ca^{2+} ion channel activity, promoting the release of Ca^{2+} from intracellular Ca^{2+} stores. Such rise in Ca^{2+} by NO was blocked by antagonists of guanylate cyclase and cADPR indicating that the downstream action of NO is mediated by both cADPR and cGMP. Parallely, the rise in cytosolic free Ca^{2+} inactivates K^+_{in} channels (blocking K^+_{in} currents) and activates Cl^- ion channels (increasing anion currents), and both events lead to stomatal closure (Garcia-Mata et al., 2003; Sokolovski and Blatt, 2004; Sokolovski et al., 2005). A possible scheme of the signal transduction mechanism involving various components is presented in **Figure 1**.

Besides their key roles during the rise in NO and subsequent effects, several signaling components tend to interact (**Table 3**). The best and well known interactions of NO are with ROS, Ca^{2+} and PA, and to some extent, with pH. For e.g., Ca^{2+} stimulates NO production and NO in turn can rise Ca^{2+} levels (Garcia-Mata and Lamattina, 2007). Such dual role of Ca^{2+} is extremely interesting and warrants detailed examination. Similarly, the production of NO and PA promote the levels of each other (Zhang et al., 2009b). There may be a feedback regulation by NO of cytosolic pH, since the rise in NO by SNP increased also the pH of guard cells (Gonugunta et al., 2008, 2009), but there is no

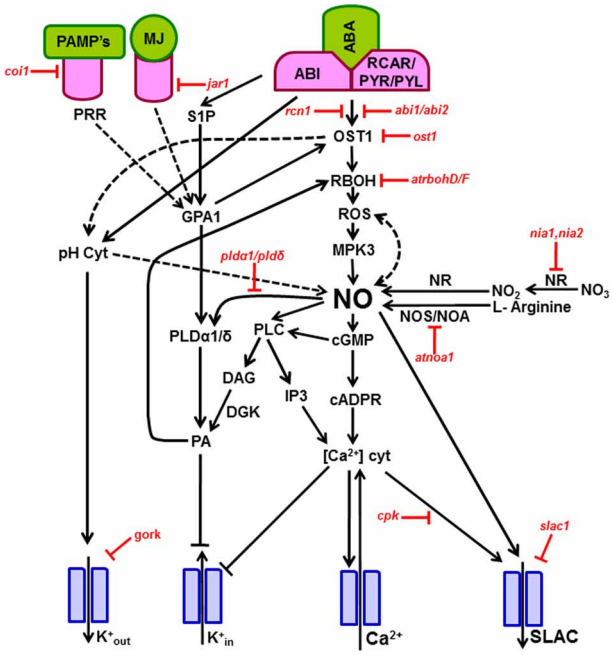

FIGURE 1 | Signal transduction mechanism involved during stomatal closure induced by ABA, MJ, and microbial elicitors. The components/secondary messengers induced by either ABA or MJ or elicitors leading to the production of nitric oxide are indicated by forward arrows. The ion channels are represented by blue color. During stomatal signaling mechanism the guard cells upon perception of ABA, MJ, or elicitors, activate NADPH oxidase, leading to a burst of ROS, which leads to a NO burst. The elevation of NO raises the cytosolic free Ca^{2+}, through up-regulation of cADPR and cGMP. In turn, the high cytosolic Ca^{2+} causes a down-regulation of K^+ inward channels and activation of outward anion channels, all leading to stomatal closure. Parallely, NO can increase the levels of PA via modulation of PLD and PLC. Several of these steps are validated by the use of mutants of *Arabidopsis* (indicated by red color), deficient in a particular signaling component. In the mutants, the relevant steps are blocked. The Arabidopsis mutants represented in this Figure are: *abi1/abi2*, ABA-insensitive (ABI1 and ABI2 protein phosphatases); *atrbohD/F*, *A. thaliana* NADPH oxidase catalytic subunit D/F; *atnoa*, *A. thaliana* nitric oxide-associated 1; *coi1*, coronatine-insensitive 1 mutant; *cpk*, calcium-dependent protein kinase; *gork*, guard cell outward rectifying K^+ channel; *jar1*, JA response 1 mutant; *nia1, nia2*, Nitrate reductase double mutant; *ost1*, open stomata 1 kinase; *pldα1/pldδ*, phospholipase α1/phospholipase δ double mutant; *rcn1*, protein phosphatase 2A regulatory A subunit 1; *slac1*, slow anion channel-associated 1 mutant. A description of these components is given in the section on "Signaling components in guard cells during NO action." Further information can be seen in **Tables 1**, **2**. Abbreviations are listed in first page. The events demonstrated by experimental evidence are represented by solid arrows. The possible interactions/effects are indicated by broken arrows.

Table 3 | Interactions of signaling components with NO during modulation of stomatal closure induced by different effectors.

Signaling component	Type of interaction	Plant	Effector	References
Cytosolic pH	Precedes NO production	*Pisum sativum*	ABA, MJ and Chitosan	Gonugunta et al., 2008, 2009
		Arabidopsis thaliana	Ethylene	Jing et al., 2010
H_2O_2	Promotes NO production	*P. sativum*	Chitosan	Srivastava et al., 2009
		A. thaliana	ABA	Bright et al., 2006
Ca^{2+}	Increases NO production	*Vicia faba*	ABA	Garcia-Mata and Lamattina, 2007
PLDα1	Increases NO production	*A. thaliana*	ABA	Zhang et al., 2009b
PLDδ	Acts downstream of NO	*A. thaliana*	ABA and NO	Distéfano et al., 2012
H_2S	Depletes NO levels in guard cells	*A. thaliana*	H_2S	Lisjak et al., 2010
	Functions downstream of NO	*V. faba*	Ethylene	Jing et al., 2012
ABA	NO increases the sensitivity to ABA	*A. thaliana*	NR and NOA	Lozano-Juste and León, 2010
MJ	Elevates endogenous ABA	*A. thaliana*	Methyl jasmonate	Ye et al., 2013

convincing evidence of such regulation of guard cell pH by NO during stomatal closure.

The marked interactions between signaling components, involving NO, constitute a dynamic and complex regulatory network. Because of the complicated nature of signaling network and strong interactions among them, only a few attempts have been made to model these events. Li et al. (2006) presented a dynamic model of signaling components in which NO is produced by NR and NOS-like enzyme, in response to ABA, and the Ca^{2+} mobilized from intracellular sources, could induce stomatal closure. Similarly, Beguerisse-Díaz et al. (2012) proposed a model of interactions between NO and ethylene. These models need to be validated by experimental evidences.

CONCLUDING REMARKS

The available literature amply demonstrates that NO is a common signaling component and a converging step for events initiated by ABA, MJ, or elicitors. The upstream components of NO, which rise during ABA action, are broadly understood. For example, ABA binds to PYR/PYL/RCAR proteins and then to PP2C forming a trimeric complex. Due to the non-availability of PP2C, protein kinases are activated to trigger several downstream elements (Cutler et al., 2010; Raghavendra et al., 2010). However, the mechanism of reception and transduction of elicitor signals, particularly the elicitor-receptor interactions, and events leading to NO rise, are not clear and need detailed examination. The levels of NO in guard cells during stomatal closure are usually monitored by using suitable fluorescent dyes, such as DAF-2DA. But these measurements are being debated, since the specificity of fluorescent dyes has been questioned, due to their proneness to artifacts. Efforts are on to reassess and reconcile measurements of NO in plant tissues (Mur et al., 2011). The exact source of NO in plant tissues continues to be a controversial topic. Several possibilities have been identified, such as NR, NIR, NOS-like and even NOA, but the available literature is not convincing enough to assess the relative significance of the different sources (Neill et al., 2008; Gupta et al., 2011a).

A range of highly interesting topics are emerging, studies on which can be quite useful. Among these are: modulation of NO by endogenous plant hormones, such as ABA (Lozano-Juste and León, 2010), role and interaction with other gaseous molecules such as H_2S and CO, termed gasotransmitters (García-Mata and Lamattina, 2013), and the post-translational modification of downstream proteins by NO or ROS or both (Yoshioka et al., 2011). In summary, further detailed work on the role and source of NO in guard cells promises to be a rewarding exercise and may provide information relevant to other plant tissues.

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S-nitrosoglutathione reductases are low-copy number, cysteine-rich proteins in plants that control multiple developmental and defense responses in Arabidopsis

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S-nitrosoglutathione reductase (GSNOR) is believed to modulate effects of reactive oxygen and nitrogen species through catabolism of S-nitrosoglutathione (GSNO). We combined bioinformatics of plant GSNOR genes, localization of GSNOR in *Arabidopsis thaliana*, and microarray analysis of a GSNOR null mutant to gain insights into the function and regulation of this critical enzyme in nitric oxide (NO) homeostasis. GSNOR-encoding genes are known to have high homology across diverse eukaryotic taxa, but contributions of specific conserved residues have not been assessed. With bioinformatics and structural modeling, we show that plant GSNORs likely localize to the cytosol, contain conserved, solvent-accessible cysteines, and tend to be encoded by a single gene. *Arabidopsis thaliana* homozygous for *GSNOR* loss-of-function alleles exhibited defects in stem and trichome branching, and complementation with Green fluorescent protein (GFP) -tagged GSNOR under control of the native promoter quantitatively rescued these phenotypes. GSNOR-GFP showed fluorescence throughout Arabidopsis seedlings, consistent with ubiquitous expression of the protein, but with especially high fluorescence in the root tip, apical meristem, and flowers. At the cellular level we observed cytosolic and nuclear fluorescence, with exclusion from the nucleolus. Microarray analysis identified 99 up- and 170 down-regulated genes ($\geq$2-fold; $p \leq 0.01$) in a GSNOR null mutant compared to wild type. Six members of the plant specific, ROXY glutaredoxins and three BHLH transcription factors involved in iron homeostasis were strongly upregulated, supporting a role for GSNOR in redox and iron metabolism. One third of downregulated genes are linked to pathogen resistance, providing further basis for the reported pathogen sensitivity of GSNOR null mutants. Together, these findings indicate GSNOR regulates multiple developmental and metabolic programs in plants and offer insight into putative routes of post-translational GSNOR regulation.

Keywords: S-nitrosoglutathione (GSNO), S-nitrosoglutathione reductase (GSNOR), nitrosative stress, trichomes, nitric oxide homeostasis, formaldehyde metabolism, glutaredoxin, pathogen defense

INTRODUCTION

In plants, biological processes ranging from leaf stomatal closure to auxin perception in roots and pathogen infection involve nitric oxide (NO) (Neill et al., 2002; Floryszak-Wieczorek et al., 2007; Lozano-Juste and Leon, 2011; Terrile et al., 2012). While NO itself is ostensibly active, it is also thought to be transmitted to distal targets via low molecular weight S-nitrosothiols (SNOs), of which the glutathione (GSH) adduct S-nitrosoglutathione (GSNO) is the most abundant (Broniowska et al., 2013; Corpas et al., 2013). GSNO can profoundly affect protein activity through glutathionylation and nitrosation of cysteines (Romeo et al., 2002; Giustarini et al., 2005; Zaffagnini et al., 2013), implying that cells require mechanisms to spatiotemporally control GSNO levels. Catabolism of GSNO by S-nitrosoglutathione reductase (GSNOR) is common to eukaryotes and many bacteria and is believed to be responsible for this regulation of GSNO levels (Liu et al., 2001; Staab et al., 2008). GSNOR exhibits NAD/H-dependent oxidoreductase activity toward a broad spectrum of aliphatic compounds, but its preferred substrates are GSNO and S-hydroxymethylglutathione (HMGSH), an intermediate in formaldehyde metabolism (Jensen et al., 1998; Achkor et al., 2003; Kubienová et al., 2013). While GSNO catabolism has been observed with Cu-Zn superoxide dismutase, GSH peroxidase, xanthine oxidase, and human carbonyl reductase 1 (CR1), the former three enzymes merely regenerate NO [summarized in Broniowska et al. (2013)], and residues critical to interaction between CR1 and GSH adducts are not conserved in plants (Bateman et al., 2008). GSNOR is therefore considered the primary catalyst for GSNO catabolism in plants.

The importance of GSNOR to plant growth, development and stress responses has been highlighted by several studies. Lowered *GSNOR* expression in *Arabidopsis thaliana*

(Arabidopsis), resulting from a null mutation (*atgsnor1-3/hot5-2*) or RNAi, was correlated with higher SNO content and differential susceptibilities to pathogens (Feechan et al., 2005; Rustérucci et al., 2007). The effect of absence of GSNOR was extended by Lee et al. (2008) who described a thermotolerance defect that was rescued with NO scavengers. Other phenotypes of plants with GSNOR mutations include diminished fertility and resistance to programmed cell death induced by paraquat, an herbicide that elicits robust reactive oxygen species (ROS) production (Lee et al., 2008; Chen et al., 2009). These concomitant gains and losses of function are analogous to consequences of GSNOR inhibition in mammals, for which both enhanced carcinogenesis and abated severity of inflammatory diseases are observed (Wei et al., 2010; Sun et al., 2011; Tang et al., 2013). Such pleiotropy suggests GSNOR participates in both homeostatic maintenance and biotic and abiotic stress responses.

The evolutionary conservation of GSNOR is high (Liu et al., 2001), and although the consequences of *GSNOR* depletion have been described at the organismal level for Arabidopsis (Lee et al., 2008; Chen et al., 2009; Kwon et al., 2012), a molecular etiology for the *GSNOR* loss-of-function phenotype is lacking. Here we sought to address the issues of how GSNOR activity could be regulated and of what processes are impacted by changes in GSNO levels and, therefore, potentially regulated by nitrosation or glutathionylation of protein effectors. We searched for conserved and unique features of plant GSNOR proteins, localized GSNOR at the tissue and cellular levels, and measured global changes in the transcriptome of an *atgsnor/hot5* null mutant in Arabidopsis. Our data demonstrate that most sequenced green plant genomes are predicted to encode a single copy of GSNOR characterized by a high content of positionally-conserved cysteines. GSNOR is found in the cytosol and nucleus throughout the plant, and is thus available to modulate GSNO concentration in most if not all cells. Moreover, alterations in the transcriptome of Arabidopsis homozygous for the *atgsnor1-3*/*hot5-2* null allele exhibited dysregulated expression of pathogen response and calcium signaling genes, but higher expression of a subset of glutaredoxin (GRX)-encoding genes. Together, these data suggest GSNOR facilitates multiple homeostatic and stress adaptation processes in green plants.

MATERIALS AND METHODS

AMINO ACID SEQUENCE ALIGNMENTS AND ANALYSIS

Predicted genes encoding GSNOR from green plants were retrieved from NCBI Genbank and Phytozome v9.1 (Goodstein et al., 2012) using Arabidopsis GSNOR (At5g43940, ADH2) as the tblastn query. *GSNOR* copy number was assessed with Phytozome and NCBI tblastn algorithms by querying predicted GSNOR- encoding genes against genomic reads from a particular plant species. Similar hits were considered duplicates if 5′ and 3′ intragenic and intron nucleotide sequences were >99% identical. Predicted GSNORs were also aligned via ClustalW (Larkin et al., 2007) with the Arabidopsis protein most similar to GSNOR (alcohol dehydrogenase, AtADH1, At1g77120), and sequences that cladded with AtADH1 were culled. Bacterial, metazoan, and fungal orthologs discussed in the text were uncovered through an NCBI tblastn search with *E. coli*, *Saccharomyces cerevisiae*, and human GSNOR queries, respectively. ClustalW sequence alignments were made with Jalview (Waterhouse et al., 2009). Deduced protein sequences were included in **Figure 1** if transcript evidence (i.e., RNAseq and/or ESTs spanning the coding sequence) was available through Phytozome. Phylogenetic trees were drawn from aligned sequences in EvolView (Zhang et al., 2012). N-terminal targeting peptide searches were performed with Predotar (Small et al., 2004) and MITOPROT (Claros, 1995). Mitochondrial targeting peptides encoded by *GSNOR* 5′ intragenic regions were identified by six-frame translation of the first 500 base pairs upstream of the start codon using the intron splice rules for Arabidopsis (Hebsgaard et al., 1996).

GSNOR STRUCTURAL ASSESSMENTS

Crystal structures of GSNOR from tomato (4DL9), human (1MP0), and Arabidopsis (4JJI and 4GL4) were obtained from the Protein Data Bank. Graphics were made with PyMOL v 1.6 (PyMOL).

PLANT MATERIAL AND GROWTH CONDITIONS

The Arabidopsis GSNOR null mutants, *hot5-2* (also known as *atgsnor1-3*) (Col-0 background) and *hot5-4* (WS background), have been described previously (Lee et al., 2008). Unless otherwise indicated, plants were grown in soil in growth chambers on 16 h days (150 μMol m^{-2} s^{-1} light intensity) and a 21/19°C day/night temperature cycle. For analysis of the number of branches produced by wild-type and mutant plants, plants were grown as above and their height and branch pattern and numbers were measured 8 weeks after germination.

GENERATION AND VISUALIZATION OF GSNOR-GFP FUSIONS IN PLANTS

Green fluorescent protein (GFP) was fused in frame 3′ to the Arabidopsis *GSNOR* genomic DNA (including 754 bp and 180 bp of 5′ and 3′ UTR, respectively) as follows. Genomic DNA was cloned into pENTR/D TOPO (Life Technologies). GFP was obtained from pMDC83 (Curtis and Grossniklaus, 2003). *Hind*III and *Xba*I sites were added to GFP and GSNOR-encoding sequences with primers GGATGCAAGCTTAGTAAAGGAGAAG AAC and TCTCTAGATTATTTGTATAGTTCATCCATGC (GFP) and primers CGTTGTG TCCTCGATACCAGCAAGCTTGTC TCTAGATGACTATATGGGTCCTCTCTGC and GCAGAGA GGACCCATATAGTCATCTAGAGACAAGCTTGCTGGTATCG AGGAC ACAACG (GSNOR). PCR products were digested with *Hind*III and *Xba*I, purified, and ligated. The GSNOR-GFP clone was then subjected to PCR with primers GGTACCGAA TTC CTAGAGTACAACCTC and TCGAGTGCGGCCGCTAAACT AT ATGATTAG to add *Eco*RI and *Not*I sites, respectively. The PCR product was then ligated into *Eco*RI/*Not*I-digested pENTR2B. Constructs were cloned into the binary vector pBIB Basta-GWR (Gou et al., 2010) with LR Clonase II (Invitrogen), after which the sequence was verified.

The GSNOR-GFP translational fusion was then transformed into *hot5-2* plants, and homozygous transformants were identified by Basta screening and western blots. Three independent transgenic lines were analyzed. Whole seedlings and roots were imaged 7 days after germination on minimal nutrient medium

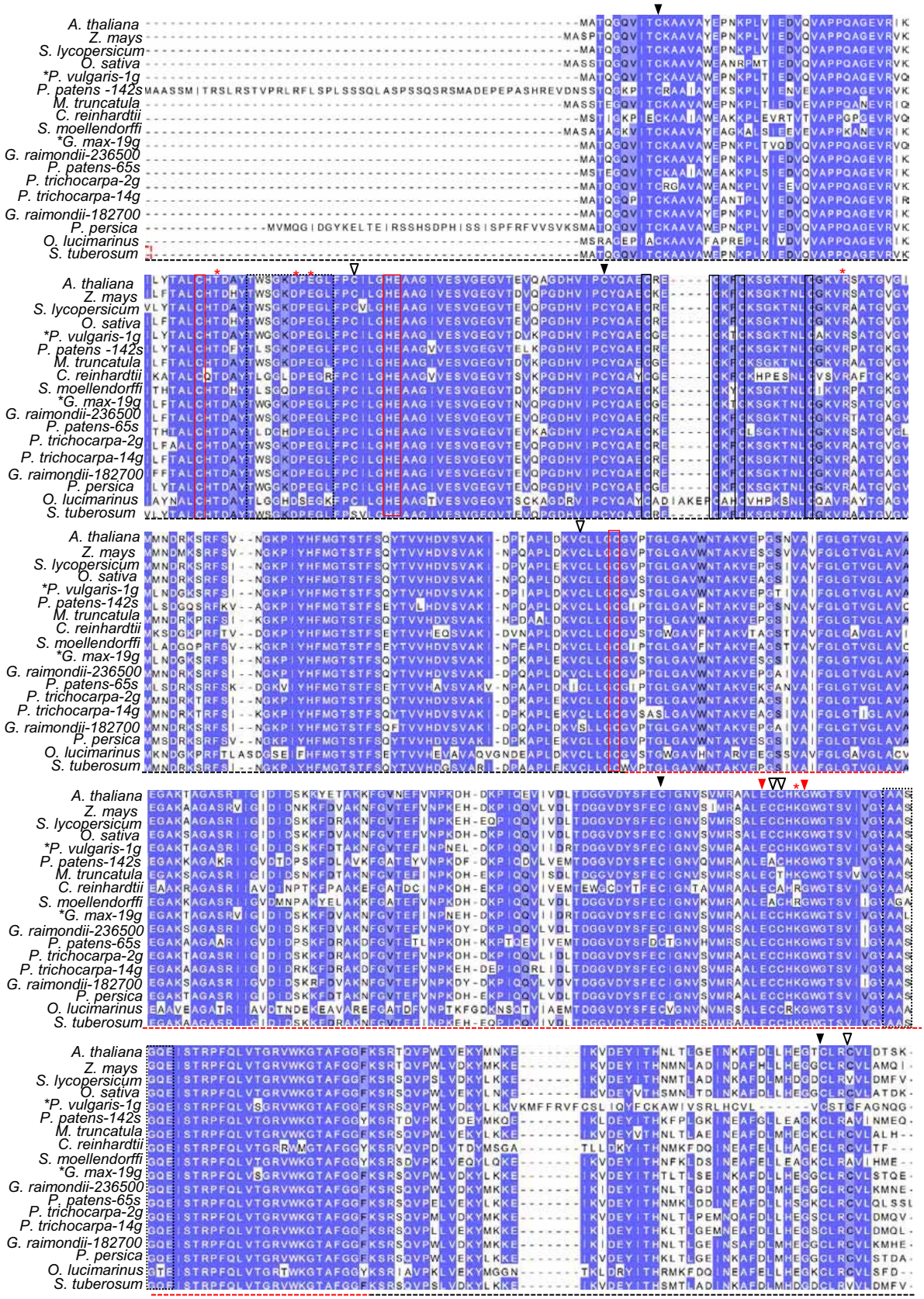

FIGURE 1 | Continued

FIGURE 1 | Continued
Conserved features of plant GSNOR proteins. Dark blue, light blue, and uncolored residues, respectively, refer to 100%, ≥75%, and <75% sequence conservation. Black and red dotted horizontal lines demarcate the catalytic and NADH-binding domains, respectively, as reported in crystal structure of tomato GSNOR (PDB code 4DL9). Residues coordinating structural and catalytic zinc atoms are outlined by solid black and red boxes, respectively. Red asterisks denote substrate-binding amino acids according to Kubienová et al. (2013). Dotted black boxes highlight flexible regions enclosing the active site. Red arrowheads (▼) indicate positions of the *hot5-1* and *hot5-3* missense mutations (Lee et al., 2008). Open (∇) and closed (▼) black arrowheads designate ex-zinc cysteines found in most and all plant sequences, respectively. *Z. mays*: maize. *S. lycopersicum*: tomato. *O. sativa*: rice. *P. vulgaris*: bean. *P. patens*: Physcomitrella. *M. truncatula*: Medicago. *C. reinhardtii*: Chlamydomonas. *S. moellendorffii*: Selaginella. *G. max*: soybean. *G. raimondii*: cotton. *P. trichocarpa*: poplar. *P. persica*: peach. *O. lucimarinus*: Ostreococcus. *S. tuberosum*: potato. *S. cerevisiae*: budding yeast. *H. sapiens*: human. Black asterisks: species with genomes predicted to encode additional paralogs, but only transcript-supported sequences are shown. Accession numbers can be found in Supp. Table 1 and an alignment of additional plant sequences not currently supported by transcript data is presented in Supp. Figure 1.

with 0.5% sucrose. Whole flowers and reproductive structures were analyzed using stage 13 or 14 flowers as indicated in the figure legend. Images were obtained using an Olympus Fluoview FV1000 confocal microscope, with the exception of the whole seed and isolated stamens, which were imaged using conventional fluorescence microscopy with a NIKON Eclipse E800 microscope equipped with a SPOT camera (Molecular Diagnostic).

MICROARRAY ANALYSIS

Wild-type (Col) and *hot5-2* Arabidopsis plants were grown on soil in a growth chamber on a 12 h light, 21°C/12 h dark, 19°C cycle for 25 days after germination. Four biological replicates of wild-type and *hot5-2* leaves were sampled for RNA extraction 1 h before the end of the light period. A two-color, dye swap hybridization was performed on a long-oligonucleotide array chip by the Galbraith lab (University of Arizona) according to published methods (Zanetti et al., 2005; Zhang et al., 2008). Data were analyzed with Robin Version 0.9.6 BETA (Lohse et al., 2010). Differentially-expressed genes were identified by two criteria: (1) change in expression greater than 2-fold, and (2) a *t*-test *p*-value <0.01. Normalization of expression data, analysis using a linear model, and Benjamini and Hochberg false discovery rate correction for multiple comparisons were performed using LIMMA (Smyth and Speed, 2003).

RESULTS

CATALYTIC AND ZINC-COORDINATING RESIDUES ARE CONSERVED IN GSNOR FROM GREEN PLANTS

To identify potentially novel shared motifs in plant GSNORs, we employed the tblastn algorithms of Phytozome (Goodstein et al., 2012) and NCBI Gene Expression Omnibus (Altschul et al., 1997) to search for GSNOR sequences supported by transcriptional data. Eighteen unique, type III ADH-encoding cDNAs were obtained from a variety of monocots, dicots, mosses, and protists. Residues near the dimer interface of tomato GSNOR (Kubienová et al., 2013), whose mutation compromises thermotolerance in dark grown Arabidopsis seedlings (Lee et al., 2008), are notably found in all plant sequences, while a glycine that when mutated to aspartate diminishes GSNOR activity, but confers enhanced paraquat resistance (Chen et al., 2009), is present in all but two algae (**Figure 1**). Substrate- and NADH-enclosing clefts (**Figure 1**, black dotted boxes) are identical or contain conservative substitutions among moss and algal orthologs. Structural and catalytic zinc-coordinating residues (**Figure 1**, solid black and solid red boxes, respectively) and substrate-binding amino acids (red asterisks), as reported by Kubienová et al. (2013), are identical in all but two predicted proteins, which contain a single lysine to arginine, conservative substitution.

GSNOR is remarkably cysteine rich, with a mole percent cysteine of 3.84 % for the Arabidopsis protein, compared to the 1.37 % average for all proteins in the UniProtKB database (2013). Because cysteines can serve as key post-translational regulatory sites being modified by nitrosation, glutathionylation, or reversible oxidation, we analyzed the conservation of the nine extra non-zinc-coordinating cysteine residues (ex-zinc cysteines) in Arabidopsis GSNOR. Four are in all the transcript-supported plant sequences, two are substituted in one, two are substituted in two, and one differs in four organisms (**Figure 1**, solid and open black arrowheads), yielding an overall conservation of 93.8 % (i.e., on average, each cysteine is present in 17 of the 18 sequences). If additional plant genes are considered for which EST support is lacking, ex-zinc cysteine conservation is still 91.0% (Supp. Figure 1—see Supp. Table 1 for accession codes). Thus, the position and frequency of ex-zinc cysteines are highly conserved in plant GSNORs. We also examined the position of ex-zinc cysteines in the Arabidopsis GSNOR structure (PDB 4JJI, via (PyMOL) and found that three were solvent accessible (Cys-10, Cys-271, and Cys-370, **Figures 2A–C**), two of which—Cys-10 and Cys-271—are positionally conserved even in the human sequence. The structures of GSNOR from human (1MP0) and tomato (4DL9) showed similar solvent exposure of the homologous ex-zinc cysteines, suggesting these three residues may have conserved functions in regulating GSNOR activity.

MOST PLANT GENOMES ENCODE ONE GSNOR PROTEIN PREDICTED TO BE FOUND IN THE CYTOSOL

Encoded by a single gene, Arabidopsis GSNOR consists of nicotinamide cofactor-binding and catalytic domains and has two primary enzymatic activities—GSNO reductase and HMGSH dehydrogenase (Lee et al., 2008; Crotty, 2009; Kubienová et al., 2013). Interestingly, although *GSNOR* is found primarily as a single-copy gene in most other examined plant species (12 of 15 transcript-supported organisms, and 35 of 41 green plants analyzed), the dicots *Populus trichocarpa* (poplar) and *Gossypium raimondii* (diploid cotton) and the moss *Physcomitrella patens* (Physcomitrella) are predicted to have two *GSNOR*s. Although not yet supported by transcript data, gene families are also predicted in *Phaseolus vulgaris* (bean), *Glycine max* (soybean), and *Malus domestica* (apple; Supp. Figure 1). Moreover, in phylogenetic trees calculated by the average distance method

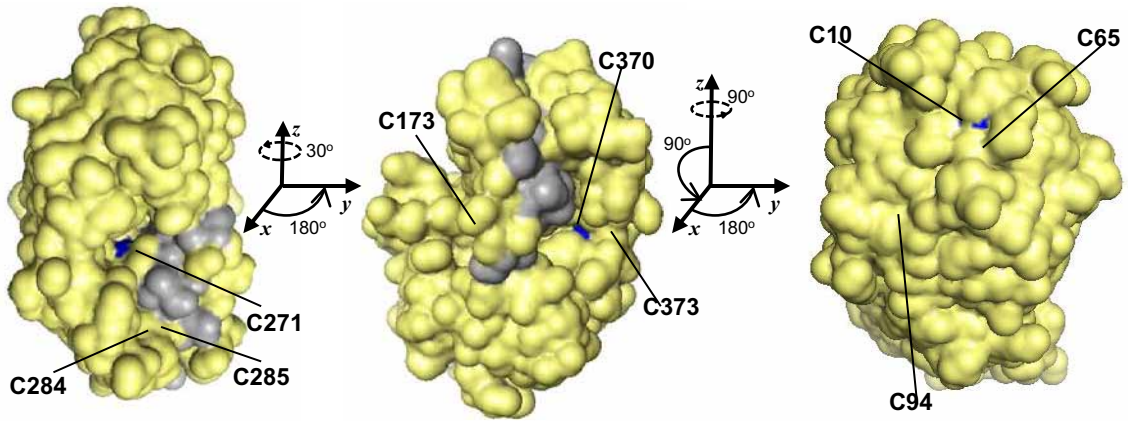

FIGURE 2 | Of nine positionally-conserved ex-zinc cysteines in GSNOR three are solvent-accessible. Three orientations (rotation angles indicated) of a monomer of the Arabidopsis GSNOR dimer are shown with solvent accessible surface (PDB 4JJI, 1.8 Å res., $R_{free} = 0.223$) in yellow. Solvent-accessible ex-zinc cysteines are indicated in blue, and the dimer interface in gray. Images were made in PyMOL.

with EvolView (Zhang et al., 2012), paralogs within the same species were more similar to one another than to orthologs in other species (data not shown). Thus, not only do most plant genomes encode one copy of GSNOR, but duplication of GSNOR-encoding genes has occurred recently and sporadically among plant species.

Extended N-termini, which could facilitate organelle targeting, are present in predicted GSNOR sequences from *Prunus persica* (peach) and Physcomitrella (**Figure 1**). Therefore, the Predotar (Small et al., 2004) program was used to search for putative endomembrane, plastid, and mitochondrial targeting peptides in all GSNORs. Of 50 input sequences, only one Physcomitrella paralog was predicted to have a mitochondrial targeting peptide. Analysis with MITOPROT (Claros, 1995) achieved similar results. Intriguingly, 5′ intragenic regions of GSNORs from Arabidopsis, rice, and *Selaginella moellendorfii* included cryptic splice sites that could give rise to putative mitochondrial targeting peptides, but intragenic regions from tomato, potato, Medicago, and *O. lucimarinus* did not. These results suggest GSNOR is a cytosolic enzyme in most plants.

We also noted predicted N- and C-terminal extensions in non-transcript-supported orthologs from two strains of *Micromonas pusilla* (an alga) and in apple that are too long to be signaling peptides (Supp. Figure 1). BLAST searches indicated *M. pusilla* extensions were formylglutathione hydrolase (FGH) domains, which catalyze the decomposition of S-formylglutathione (FGSH) to GSH and formate. FGHs are predicted in many green plants, albeit as separate gene products. FGSH is the product of the HMGSH dehydrogenase activity of GSNOR and is a key intermediate in formaldehyde detoxification (Staab et al., 2008). This observation suggests that, at least in the case of *M. pusilla* strains, NO and formaldehyde metabolism are intimately linked. The C-terminus of an apple GSNOR comprises a 4,5-DOPA dioxygenase (DOD), an enzyme in the biosynthesis of phenylpropanoid-derived betalain pigments that aid in plant defense (Georgiev et al., 2010), but transcriptional support has not been obtained for this fusion.

EXPRESSION AND LOCALIZATION OF ARABIDOPSIS GSNOR

To determine the major sites of GSNOR function, we created a translational fusion of GPF at the C-terminus of GSNOR driven by native promoter sequence and transformed this construct into the *GSNOR* null mutant *hot5-2* (Lee et al., 2008). We carried forward three homozygous transgenic lines that express GSNOR-GFP (**Figure 3A**). Mutation of *GSNOR* results in reduced plant height and an increased number of inflorescences (Lee et al., 2008; Kwon et al., 2012). We quantified these differences in mature plants and found that while *hot5-2 and hot5-4* null mutants and corresponding wild-types (Col-0 and WS, respectively) produce a similar number of first-order inflorescence stems (arising from the rosette), mutants produce two-fold more second-order branches and often produce third-order branches, which are not observed in wild-type plants (**Figures 3B–D**). All three complemented lines showed quantitative restoration of wild-type height and branching patterns (**Figures 3C,D**), indicating that the GFP fusion did not compromise GSNOR function. We further observed reduced trichome branching in *hot5* null plants (**Figures 3E,F**), a previously unreported phenotype. Mutants primarily produced trichomes with only two branches, rather than the three or four typical of wild type (**Figure 3F**). 70% of *hot5-2* trichomes were two-branched compared to virtually zero in Col, and similar differences were detected between WS and *hot5-4* (**Figure 3G**). The absolute number of trichomes was marginally higher in WS leaves compared with those of *hot5-4*, in agreement with Holzmeister et al. (2011), while trichome abundance did not differ between Col and *hot5-2* (**Figure 3E**, lower panel), indicating the difference in trichome number must be due to a modifying gene present only in the WS background. Quantitation of trichome branching in the complemented lines showed that the GSNOR-GFP fusion protein restored branching to wild-type levels (**Figures 3F,G**).

Based on complementation of *hot5* phenotypes, the GFP fusion seemed suitable to assess GSNOR localization. Whole seedlings at the cotyledon stage and flower stage 13 were

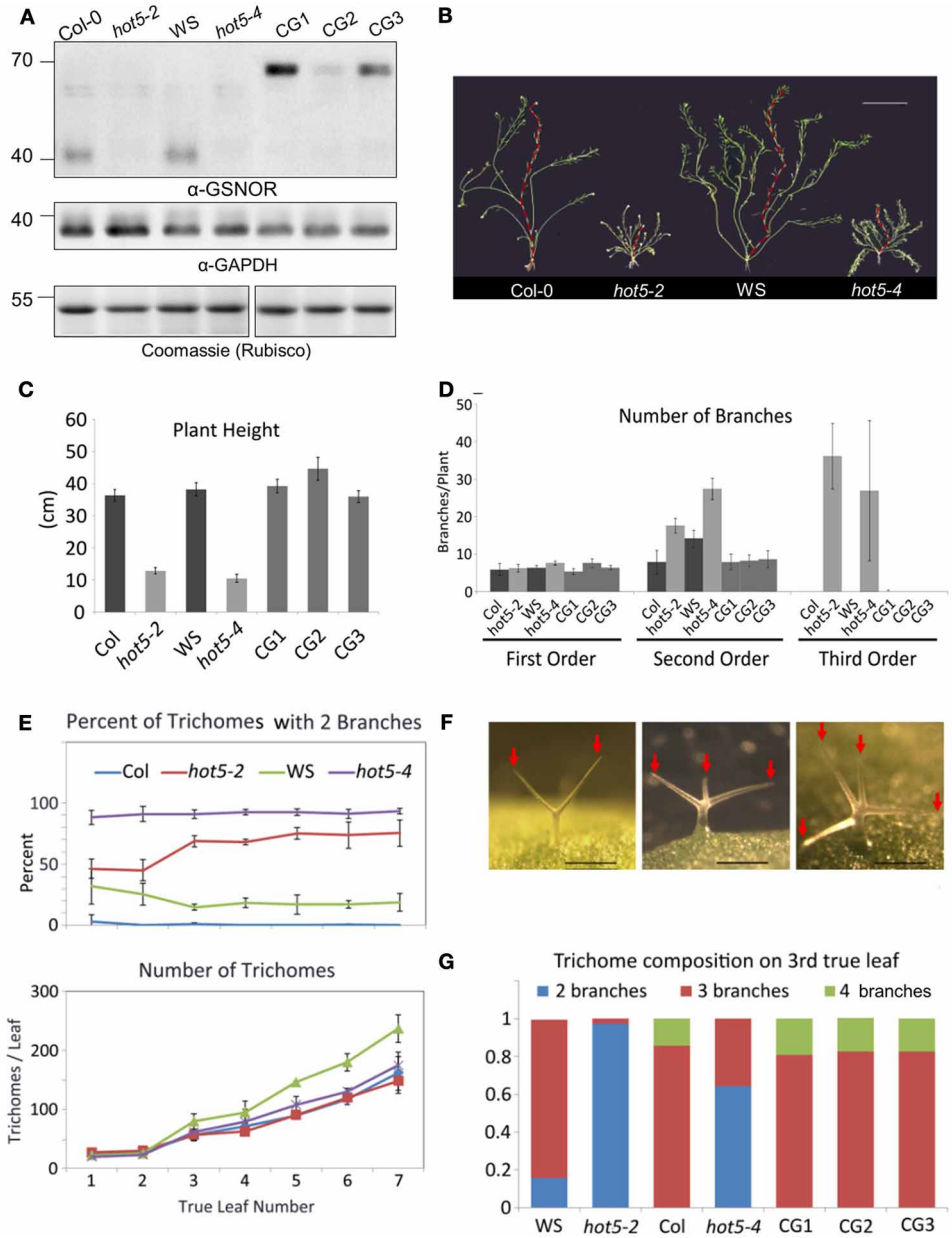

FIGURE 3 | Expression of GSNOR-GFP under its own promoter rescues multiple aspects of the *hot5-2* null phenotype. (A) Immunoblots of leaf protein extracts probed with anti-Arabidopsis GSNOR antibodies (top) or anti-cytosolic GAPDH blots (middle). Rubisco large subunit (Coomassie stain, bottom). Plant genotypes are as indicated with CG1, 2, and 3 being independent homozygous T3 lines expressing GSNOR::GSNOR-GFP in the *hot5-2* background. **(B)** Shoot systems of indicated genetic backgrounds with primary inflorescence overlaid in red. Bar: 5 cm. **(C,D)** Shorter plant height **(C)** and inflorescence branching order **(D)** are rescued to wild-type levels in CG1, 2, and 3. **(E)** Trichomes with reduced branching are more numerous in *hot5-2* and *hot5-4* rosette leaves than in respective wild-type backgrounds. Error bars: *STD* **(F)** Left to right: trichomes from *hot5-2*, Col, Col. Bar: 0.2 mm. Red Arrows indicate trichome branches. **(G)** Trichome branching is rescued to wild-type levels in CG1, 2, and 3.

imaged to observe the tissue and subcellular localization of GSNOR-GFP (**Figure 4**). Fluorescence was observed throughout seedling and floral structures (**Figures 4A–C**), which correlates with gene expression data in public databases (Toufighi et al., 2005). The seedling apical meristem and root tip exhibited notably intense fluorescence (**Figures 4A,D**), while cotyledon, hypocotyl, root, and petal vascular tissue signals were highest (**Figures 4A–C**). Detailed observation of sections through the root tip (**Figures 4E–G**) show distribution in all root cell types with diffuse cytosolic and nuclear localization, but dramatic exclusion from the nucleolus. This is also evident in the elongating zone of the root, although nucleoli are considerably smaller in these cells (**Figure 4H**). GSNOR-GFP could also be detected in anther filaments, ovary, stigma, and petals (**Figures 4I,J,M,N,P**)

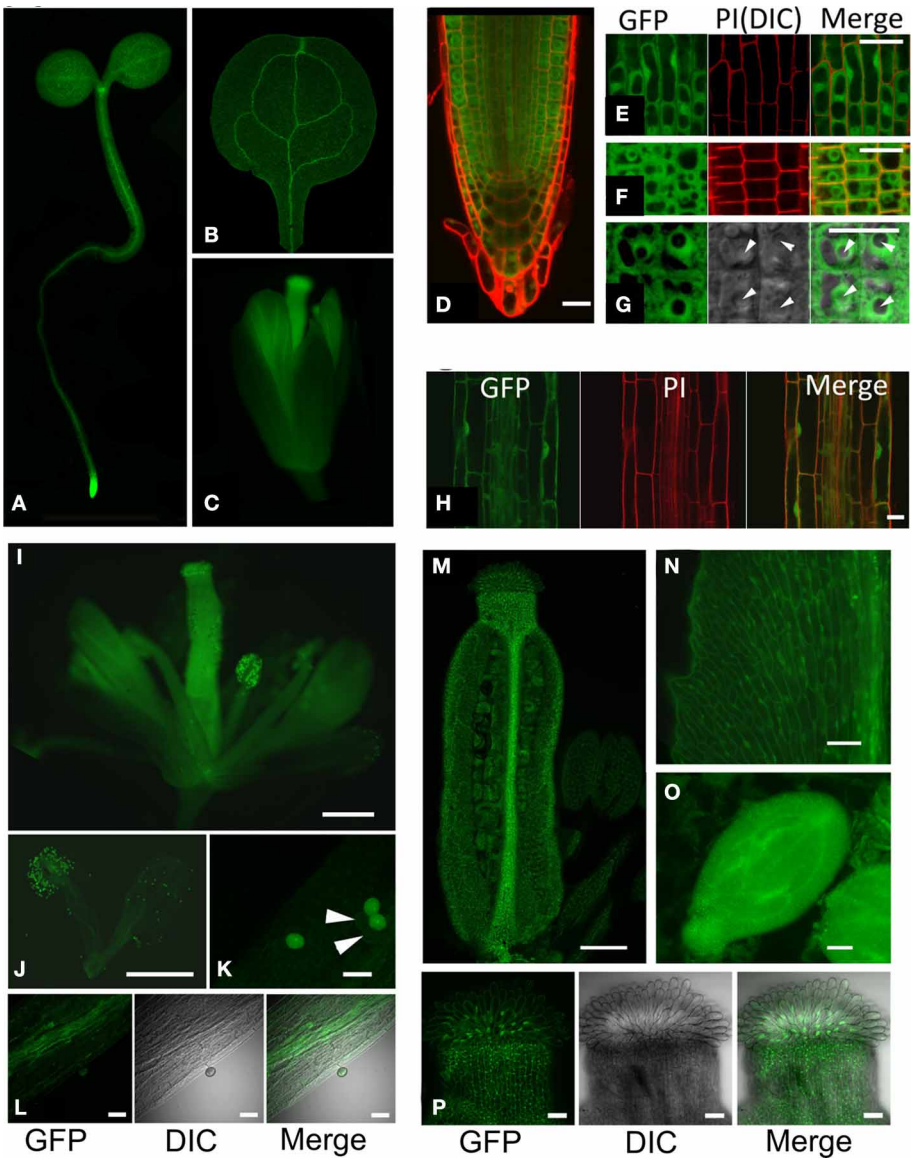

FIGURE 4 | GSNOR is expressed in various organs and developmental stages in Arabidopsis. Localization of GSNOR was observed in plants transformed with GSNOR::GSNOR-GFP in the *hot5-2* background. All plants were homozygous for the GSNOR::GSNOR-GFP transgene, with the exception of those used for images in **(I–K)**. **(A)** Whole seedling. **(B)** Cotyledon. **(C)** Flower, stage 13. **(D)** GSNOR-GFP distribution and localization in root tip cells in optical cross section through the middle of the root. **(E)** GSNOR-GFP localization in root epidermal cells. **(F, G)** Optical cross section of GSNOR-GFP localization in root cortex cells at two different magnifications. Arrowheads: nucleolus. PI, Propidium Iodide staining; DIC, Differential Interference Contrast microscopy. **(H)** GSNOR-GFP localization in the root elongation zone. Bar: 20 μm **(D–H)**. **(I–P)** Localization of GSNOR-GFP in stage 14 flowers **(I)** stamens and petals **(J)** pollen **(K)** anther filaments **(L)** ovary **(M)** petals **(N)** seed at bending cotyledon embryonic stage **(O)** and stigma **(P)**. Arrowheads in **(K)** denote *hot5-2* pollen that do not express GSNOR-GFP due to segregation of the transgene in the heterozygote. Bar: 500 μm **(I,J)** 200 μm **(M)**, or 40 μm **(K,L, N–P)**.

of stage 14 flowers and was particularly enriched in pollen and seed (**Figures 4K,O**).

MULTIPLE PATHOGEN RESPONSE GENES ARE DOWNREGULATED IN *hot5-2*

To assess global changes in the transcriptome due to GSNOR absence, we performed microarray analysis on 4-week leaves of *hot5-2* plants grown on a 12 h light cycle since the leaves of Col and *hot5-2* plants are most morphologically comparable at this age (Lee et al., 2008). Imposing stringent criteria of $\geq$2-fold changes with $p \leq 0.01$ (FDR-corrected), we found 99 and 170 transcripts up- and downregulated, respectively, in *hot5-2* compared to Col-0. A complete list of transcripts is provided in Supp. Table 2 categorized by pathway as curated in MAPMAN (Usadel et al., 2009). This list was used to test for enriched categories of regulated genes (Usadel et al., 2006).

One group of enriched genes was the "Stress Response" category (**Table 1**). Of 19 genes in this category, 13 were down-regulated "biotic stress" genes, including pathogenesis-related protein 1 (PR1, ∼6.9-fold), consistent with data from Feechan et al. (2005), four potential pathogen receptors (At1g59218, At3g04210, At3g50470, At3g11010), PR5, and other predicted herbivore and pathogen defense proteins. Of two upregulated "Stress" category genes, the defensin protein PDF1.2 was also reported to be upregulated in *GSNOR* antisense plants (Espunya et al., 2012).

These results are consistent with the involvement of NO in pathogen responses, and prompted us to employ the Arabidopsis eFP browser (Toufighi et al., 2005) and TAIR gene annotations to determine if other pathogen response-linked genes, including those in the "Not Assigned" category (the largest category with 76 genes) were also differentially regulated in *hot5-2*. The eFP data include response to the oomycete *Phytophthora infestans*, the bacterium *Pseudomonas syringae* (virulent and avirulent), the fungi *Botrytis cinerea* and *Erysiphe orontii*, as well as induction by non-host bacteria and several elicitors. As highlighted in Supp. Table 2, 43 additional genes that can be linked to pathogen response are down-regulated (e.g., ACD6, At4g14400; DIR1, At5g48485), making a total of 56 of 170 downregulated genes linked to pathogen response. In contrast, only 13 additional upregulated genes can be linked to pathogen response. Since ∼12% of Arabidopsis genes are pathogen defense-related (The Arabidopsis Initiative, 2000), we conclude the absence of *GSNOR* disproportionately downregulates pathogen response genes.

AN UNUSUAL CLASS OF GLUTAREDOXINS ARE UPREGULATED IN *hot5-2*

Given the significant interplay between GSNO levels and GSH-controlled redox homeostasis (Staab et al., 2008), it was of interest that genes in the "Redox" category were also enriched. Intriguingly, six cytosolic ROXY-class glutaredoxins (GRXs) and an atypical chloroplast-localized thioredoxin (TRX) were upregulated in *hot5-2* (**Table 2** and Supp. Table 2). GRXs and TRXs are small oxidoreductases that regulate the thiol redox state of other proteins (Meyer et al., 2012). Four of the six up-regulated GRX genes are closely linked on chromosome 4 and share 91–95% identity, while two others on chromosomes 5 and 1 are 72–75% and 58–60% identical to the chromosome 4 genes, respectively, and 59% identical to each other. All six GRXs belong to a plant-specific GRX family containing a monocysteine active site. Thus, these GRX proteins likely act as monothiol GRXs (Herrero and De La Torre-Ruiz, 2007). One cytosolic, monothiol ROXY GRX (ROXY20), which is divergent from the upregulated genes (∼40% identical), is downregulated in *hot5-2*. Examination of publically available expression patterns of these regulated ROXY genes (Schmid et al., 2005) indicates that during normal plant growth the *hot5-2* upregulated *ROXY* genes have low transcript levels in stems, senescing leaves, and the shoot apex, while *ROXY20* shows the opposite pattern. Thus, these GRX proteins are likely to serve different functions. Currently the redox targets of these cytosolic GRXs and the unusual chloroplast TRX are unknown, but increased expression of the corresponding genes indicates that mutation of *GSNOR* alters redox homeostasis and potentially the targets of these oxidoreductases.

Table 1 | Changes in "Stress Responsive" Transcripts in *hot5-2* vs. wild-type.

Gene	Protein	Stress	Fold-change (log2)	*p*-value
UPREGULATED				
At3g04720	PR4 (PATHOGENESIS-RELATED GENE 4)	Biotic	1.397	0.001
At5g44420	PDF1.2; PDF1.2A; LCR77; Defensin	Biotic	1.017	0.001
DOWNREGULATED				
At2g14610	PR1 (PATOGENESIS-RELATED GENE 1)	Biotic	−2.78	0
At4g19820	Glycosyl hydrolase family 18 protein	Biotic	−2.25	0
At2g37570	SLT1 (SODIUM AND LITHIUM TOLERANT 1)	Abiotic	−2.005	0.001
At1g73330	DR4 (DROUGHT REPRESSED 4); peptidase inhibitor	Biotic	−1.882	0
At3g50480	HR4 (HOMOLOG OF RPW8 4)	Biotic	−1.861	0.001
At1g75040	PR5 (PATHOGENESIS-RELATED GENE 5)	Biotic	−1.821	0.001
At3g11010	AtRLP34 (Receptor-like Protein 34)	Biotic	−1.8	0.002
At1g24020	MLP423 (MajorLatexPprotein-LIKE PROTEIN 423)	Abiotic	−1.65	0
At3g50470	HR3 (HOMOLOG OF RPW8 3)	Biotic	−1.502	0
At2g43510	ATTI1; serine endopeptidase inhibitor	Biotic	−1.414	0
At3g04210	Disease resistance protein (TIR-NBS class)	Biotic	−1.391	0.001
At2g43530	Trypsin inhibitor Defensin-like protein	Biotic	−1.282	0.001
At1g59218	Disease resistance protein (CC-NBS-LRR class)	Biotic	−1.272	0.001
At3g48080	Lipase class 3 family protein	Biotic	−1.238	0.001
At1g72260	THI2.1 (THIONIN 2.1)	Biotic	−1.117	0.001
At2g03720	MRH6 (morphogenesis of root hair 6)	Abiotic	−1.083	0.008
At1g19670	COR1 (CORONATINE-INDUCED PROTEIN 1); CHL1 (CHLOROPHYLLASE 1)	Abiotic	−1.013	0.001

$p \leq 10^{-8}$ *listed as* $= 0$.

Table 2 | Changes in "Redox" Transcripts in *hot5-2* vs. wild-type.

Gene	Protein	Function/Localization	Fold-change (log2)	*p*-value
UPREGULATED				
At4g15690	Glutaredoxin S5	Glutaredoxin; cytosolic; CxxS active site	2.788	6.94 E-05
At4g15670	Glutaredoxin S7	Glutaredoxin; cytosolic; CxxS active site	2.758	8.85 E-05
At4g15660	Glutaredoxin S8	Glutaredoxin; cytosolic; CxxS active site	2.088	0
At4g15700	Glutaredoxin S3	Glutaredoxin; cytosolic; CxxS active site	1.786	0
At1g03020	Glutaredoxin S1	Glutaredoxin; cytosolic; CxxS active site	1.595	0
At5g04720	ACHT5 (ATYPICAL CYS HIS RICH THIOREDOXIN 5)	Thioredoxin; chloroplast; CGGC active site	1.128	0.001
At5g18600	Glutaredoxin S2	Glutaredoxin; cytosolic; CxxS active site	1.064	0.001
DOWNREGULATED				
At5g11930	Glutaredoxin C10	Glutaredoxin; cytosolic; CxxS active site	−1.155	0.001
At1g20620	CAT3	Catalase; peroxisome; cytosol?	−1.108	0.002

$p \leq 10^{-8}$ listed as = 0.

CHANGES IN *hot5-2* GENE EXPRESSION INDICATE OTHER PROCESSES IMPACTED BY NO HOMEOSTASIS

Another enriched category of genes that presents a consistent picture of changes in the GSNOR mutant is the "Signaling" category, in which 14 of 15 genes are down regulated and half of the downregulated genes are involved in calcium signaling, including multiple calmodulins, calmodulin-like proteins and calreticulins (Supp. Table 2). The single upregulated gene in this category is ATCP1 (CALCIUM BINDING PROTEIN 1, At5g49480), which also has sequence similarity to calmodulin. These data provide a direct link of GSNO to calcium signaling.

Although "Transcription" was not a specifically enriched category, consideration of regulated transcription factors (Supp. Table 1) shows that three of the most highly upregulated genes encode BHLH proteins involved in iron deficiency responses (BHLH100, At2g41240; BHLH039, At3g5698; BHLH038, At3g56980) (Wang et al., 2013). The potential targets of these transcription factors in leaves are unknown, although in roots they can activate genes required for iron uptake. Among downregulated genes in the transcription category, it is notable that there are three AP2/EREBP transcription factors, which are involved in ethylene responses, including the most strongly downregulated gene in this category, TINY (At5g11590; >5-fold decreased) (Sun et al., 2008).

DISCUSSION

The pleiotropy of Arabidopsis *GSNOR* loss-of-function mutants indicates that enzymatic control of GSNO levels is essential for competitive viability (Lee et al., 2008; Kwon et al., 2012) (Feechan et al., 2005). *In vitro*, GSNOR catabolizes GSNO and HMGSH, metabolites generated from NO- and formaldehyde, respectively. Because NO can lead to the generation of reactive nitrogen species (RNS) and formaldehyde can produce ROS (Staab et al., 2008; Kubienová et al., 2013), GSNOR is likely critical to regulation of downstream physiological and pathological effects of RNS and ROS in plants. The evolutionary conservation of GSNOR in eukaryotes and bacteria, which was recognized over a decade ago (Liu et al., 2001), further indicates this enzyme has similar functions in multiple domains of life. Here we have identified conserved features of *GSNOR* genes and their encoded proteins in plants, determined aspects of tissue and cellular localization of GSNOR, and shown that its absence impacts expression of pathogen response, redox, and calcium signaling genes in Arabidopsis. While this work is primarily descriptive, it offers insight into the molecular consequences of GSNOR loss and therefore can serve as a prospectus for future mechanistic studies.

GSNOR was found as a single-copy gene in 35 of 41 plant species, with seven instances of recent duplication events (data not shown). De Smet et al. (2013) theorize that the preponderance of single copy genes in organisms that have undergone whole genome duplication (such as Arabidopsis) is not random, but rather indicates that these genes impair fitness when present in multiple copies, possibly due to overly-robust activity of their encoded proteins. In any event, GSNOR copy number appears to be under strong selective pressure.

All putative eukaryotic GSNORs we examined show high conservation and are unusually rich in cysteines, which are highly positionally conserved among plant orthologs (**Figures 1**, **2** and Supp. Figure 1). Common functions of protein cysteine residues include coordination of metal atoms (i.e., copper and zinc), covalent catalysis, extracellular adhesion, and redox sensing (Wang et al., 2012). Most of the ex-zinc cysteines in Arabidopsis GSNOR were found to be inaccessible to solvent (**Figure 2**; Crotty, 2009), suggesting their primary function may be structural. Intriguingly, however, three cysteines that are positionally conserved between plants and animals are solvent accessible (**Figure 2**, blue patches). These residues may serve as sites of post-translational regulation via, for instance, glutathionylation or S-nitrosation. Residues that bind HMGSH or coordinate zinc (Kubienová et al., 2013) were found in all predicted plant proteins (**Figure 1** and Supp. Figure 1), indicating GSNO reductase and HMGSH dehydrogenase activities are probably general features of plant GSNORs. GSNORs from Arabidopsis and tomato exhibit ∼10-fold higher velocity of NADH-dependent GSNO reduction than NAD^+-dependent HMGSH oxidation to formylglutathione (Crotty, 2009; Kubienová et al., 2013), but the high ratio of NAD^+ to NADH in most living cells would favor the dehydrogenase reaction. This could be circumvented by cofactor recycling, wherein NADH produced from HMGSH oxidation

is employed for GSNO reduction (Staab et al., 2008). Thus, GSNO catabolism and formaldehyde detoxification may be partially interdependent processes. This scenario is not unreasonable, as ROS and RNS are known to contribute to the formation of one another (Molassiotis and Fotopoulos, 2011). The mitochondrion is a major site of ROS production in eukaryotes. GSH and NO concentrations are reported to be high in Arabidopsis mitochondria (Wang et al., 2010; Koffler et al., 2013), and mitochondrial enzymes have been identified as nitrosation targets (Millar and Day, 1996; Palmieri et al., 2010). There is no transcriptional evidence for mitochondrion-localized GSNORs apart from one Physcomitrella homolog (**Figure 1**), but the existence of cryptic target peptide-encoding sequences in frame with GSNORs from diverse taxa (data not shown) underscores possible mitochondrial localization in a common evolutionary ancestor.

Visualization of GSNOR-GFP provided clear evidence for cytosolic and nuclear localization of this protein throughout the plant, with potentially higher concentrations in vascular tissues and very noticeable exclusion from the nucleolus. Though GSNOR lacks a nuclear localization signal, it may be transported in association with another protein. We saw no evidence of GSNOR-GFP in mitochondria, but the upstream region with potential to encode a mitochondrial targeting peptide was not included in our construct. Our results of organ and tissue localization do not fully agree with a previous report from Espunya et al. (2006); while they found high levels of activity in roots, no activity was detected in the hypocotyl or cotyledons, which show significant fluorescence in our study. Consistent with our observations, their immunocytochemistry showed GSNOR in all cell types of the root meristematic zone. However, the inner cortex of the root elongation zone appeared to lack protein when localized by immunocytochemistry, in contrast to our observations. Overall, GSNOR appears to function in essentially all plant cell types. These protein data are augmented by publically available transcript analysis in Arabidopsis, which show virtually ubiquitous expression of GSNOR mRNA, further supporting the role of this enzyme in multiple plant processes.

A well-documented feature of GSNOR null Arabidopsis is a multi-branching phenotype (Lee et al., 2008), which has been suggested to arise from impact on auxin transport and function, and/or cytokinin signaling (Kwon et al., 2012). Replacement of wild-type GSNOR with GSNOR C-terminally fused to GFP produced plants with quantitatively normal branching patterns, indicating GSNOR-GFP effectively functions like the wild-type protein in processes required for normal branching. The GSNOR-GFP transgene also rescued reduced trichome branching, a previously unrecognized phenotype of the null mutant. Arabidopsis trichomes are single cells, so reduced branching results from alteration of cell morphogenesis, which involves a wide range of basic processes (Hülskamp, 2000). How absence of GSNOR reduces trichome branches is not known, but notably, Arabidopsis trichomes have two to four times the concentration of GSH compared to other epidermal cells (Gutiérrez-Alcalá et al., 2000). High GSH may increase the deleterious effects of the absence of GSNOR specifically in these cells. GSNOR-GPF also rescued this phenotype, implying a role for NO homeostasis in basic cell morphological processes. The pleiotropy of the *GSNOR* loss-of-function phenotype suggests enhanced nitrosation of one or more proteins interferes with their normal physiological functions. However, there are only a few known *in vivo* targets of nitrosation in plants: the Arabidopsis cytokinin signal relay kinase AHP1 is negatively regulated via nitrosation of a conserved cysteine (Feng et al., 2013), while nitrosation of the F box protein TIR1 has been proposed to modulate auxin signaling (Terrile et al., 2012). Thus, the strong morphological phenotypes of *hot5-2* and *hot5-4* plants may reflect uncoordinated cytokinin and auxin crosstalk caused by aberrant nitrosation of TIR1 and AHP1. Observed changes in pathogen sensitivity may have a similar etiology. Indeed, salicylic acid (SA) signaling has been shown to be downreguated by GSNO-mediated nitrosation of NPR1 (Tada et al., 2008). Further work with the GSNOR null mutant will be required to determine any alterations in nitrosated or glutathionylated targets.

Comparison of transcript levels between a *GSNOR* null mutant and wild type Arabidopsis on microarrays revealed an enrichment in transcripts involved in pathogen responses, redox regulation, and calcium signaling, as well as upregulation of transcription factors involved in iron responses and downregulation of ethylene responsive transcription factors. The extensive downregulation of pathogen defense related and responsive genes supports the observations of Feechan et al. (2005), who reported the null mutant had reduced R gene mediated, basal and non-host resistance to pathogens. While the Arabidopsis homolog of human CR1 was not transcriptionally higher in the *hot5-2* mutant, as might be expected if it provided compensatory activity, *GRX*s and a *TRX* were notably upregulated. TRXs and GRXs can mediate denitrosation and deglutathionylation, respectively (Benhar et al., 2009; Zaffagnini et al., 2013), such that they could function to reverse an increase in these modifications due to excess GSNO in the mutant. Although the contribution of TRXs and GRXs to the acclimation of GSNOR-deficient plants to nitrosative stress has not been assessed, these microarray data strongly implicate the involvement of GRXs in plant SNO homeostasis. The microarray data also support proposed linkages between NO and calcium signaling (Courtois et al., 2008), as five calmodulins or calcium binding proteins are downregulated and one is upregulated in *hot5-2*.

We also compared the results of our microarrays with previous studies aimed at identifying NO regulated genes that used NO donors or NO synthase inhibitors to induce changes in gene expression in Arabidopsis (Parani et al., 2004; Besson-Bard et al., 2009). We found very limited overlap with potentially NO-regulated transcripts. For genes induced by application of 0.1 or 1.0 mM SNP (Parani et al., 2004), only four genes were also upregulated in *hot5-2*: the calmodulin-like ATCP1 (At5g49480), WRKY40 (At1g80840), a UDP glucosyl transferase (At1g05560) and a 67 amino acid unknown (At4g27654). Compared to transcripts regulated by a NOS inhibitor, in which downregulated genes were proposed to be normal targets of NO, again only four transcripts behaved similarly in our experiments (DIN10, At5g20250; XTR8,At3g44990;

LTP1,At2g45780; unknown, At3g56360). We may see little overlap with these prior investigations because they examined short-term manipulation of NO levels, whereas GSNO and NO are chronically deregulated in *hot5-2*. In any event, changes in gene expression in the absence of GSNOR reflect a long-term metabolic adjustment required to cope with excess GSNO and the pathways it normally regulates.

GSNOR clearly plays a role in biotic stress adaptation, but how a GSNOR-DOD fusion such as that identified in a homolog from apple (Supp. Figure 1) would function *in vivo* is not clear, and this may be an artifact of early phase sequencing. Using the Toronto Bio-Analytic Resource (BAR) Arabidopsis gene expression data compendium (Toufighi et al., 2005), it was revealed that *GSNOR* transcription strongly correlated with NINJA [$r^2 = 0.62$, a jasmonic acid (JA) response corepressor] and PMR5 ($r^2 = 0.59$, a protein whose absence affords greater resistance to biotrophic fungi that cause powdery mildew). Both PMR5 and NINJA negatively regulate pathogen-induced defense signaling—PMR5 contributes to JA-independent fungal disease susceptibility (Vogel et al., 2004), while NINJA activity is curtailed following JA-induced, COI1-dependent proteasomal degradation of JAZ repressor proteins (Pauwels et al., 2010; Sheard et al., 2010). While the association of these pathogen response genes with *GSNOR* is only correlative, it can be inferred that GSNOR might also work to dampen biotic stress responses in the absence of elicitation. This would explain why *GSNOR* over-expression and RNAi-mediated knockdown served to respectively diminish and enhance systemic acquired tolerance and basal tolerance to a *P. syringae* and *Peronospora parasitica* (Rustérucci et al., 2007). This also harmonizes well with the observation that GSNOR positively affects SA signaling (Feechan et al., 2005), since JA and SA operate antagonistically to one another.

In summary, GSNOR appears to be a ubiquitously-expressed, cytosol-localized protein that regulates shoot morphology, pathogen defense responses, and NO homeostasis. Aberrant nitrosation of auxin, cytokinin, and SA response regulator proteins, among others, likely contribute to aspects of *hot5* null mutant phenotypes. Diminished branching in GSH-rich trichome cells further underscores the role of GSNOR in maintaining the cellular reduction potential, and its conserved, solvent-accessible cysteines may function as NO sinks or serve a regulatory role. The upregulation of transcripts of a class of GRXs is a particularly promising discovery, as some GRXs and TRXs catalyze deglutathionylation and denitrosation. Understanding how GRXs may compensate for loss of GSNOR and how GSNOR activity may be regulated through its conserved, solvent accessible cysteine residues will help to clarify the role of GSNOR in plant biology.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2013.00430/abstract

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