Towards genetically encoded sensors for nitric oxide bioimaging in planta

Vajiheh Safavi-Rizi 1,*†

1 Department of Plant Physiology, Institute of Botany and Landscape Ecology, University of Greifswald, Soldmannstrasse 15, 17487 Greifswald, Germany
*Author for communication: vajiheh.safavi-Rizi@uni-greifswald.de
†Senior author.

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Dear Editor,

Nitric oxide (NO) is a multitasking signaling molecule that is functional in a wide range of organisms such as humans, animals, plants, fungi, and bacteria (Lindermayr and Durner, 2018). In plants, NO plays a role in diverse morphological and physiological events such as seed germination, root emergence, hormone response, fruit ripening, and senescence as well as response to biotic and abiotic stresses (Simontacchi et al., 2015; Safavi-Rizi et al., 2020). Although our understanding of the role of NO in animal and plant cells is progressing rapidly, the advancement for spatio-temporal quantification of NO in vivo, particularly in plant cells, has been lagging.

The restrictions of the current methods of NO detection lay in their limited specificity and/or sensitivity and their lack of the suitability to give insight on the spatio-temporal NO dynamics inside living cells as they require sample processing (Ren and Ai, 2013). Another major obstacle is the simultaneous detection of NO in multiple compartments (Bryan and Grisham, 2007). This is particularly important considering the low half-life of NO and its high reactivity and complexity of its biosynthesis (Figure 1) in planta (Lindermayr and Durner, 2018). It is crucial to study the real-time dynamics of NO synthesis and signaling during development as well as stress response. Moreover, it is necessary to understand to what extent NO signaling patterns are specific to the type and duration of various stresses. Hence, developing genetically encoded NO sensor proteins can provide a promising tool for spatio-temporal NO detection in living single cells at the subcellular level under control and stress conditions, which is not possible with the current detection methods in plant cells.

Several genetically encoded fluorescent protein (FP) sensors have been developed for in vivo detection of NO in animals (Table 1). However, the adaptation and implementation of such sensors in plants remain limited.

Development of genetically encoded NO probes (geNOps) provides a recent advancement in NO-biosensing by an FP sensor. GeNOps has been established for in vivo NO bioimaging in animal cells but has similar potential for in planta NO bioimaging. GeNOps may enable a multifaceted approach for the detection of NO formation, diffusion, and degradation with the option of NO detection with subcellular resolution (Eroglu et al., 2016; Eroglu et al., 2017; Eroglu et al., 2018). GeNOps structure is based on GAF (cyclic GMP, adenylyl cyclase, FhlA), a NO binding domain originated from bacteria fused to FP variant (cyan, green, or orange FP; Eroglu et al., 2016). The GAF domain belongs to NorR (Anaerobic NO reductase transcription regulator), a NO-responsive transcription factor in Escherichia coli (D’Autreaux et al., 2005). In the presence of NO, it binds to the iron(II) of GAF giving rise to a NorR–[Fe(NO)]7...
complex that shows decreased fluorescence intensity (D’Autreaux et al., 2005; Figure 1). Since this system is reversible, NO decrease recovers the fluorescence intensity (Eroglu et al., 2016; Eroglu et al., 2017; Eroglu et al., 2018).

Long-standing questions in plants that could be addressed through the implementation of genetically encoded NO biosensors, such as geNOps, include

(1) To what extent does organelle-generated NO reach the nucleus to modulate gene expression under stress conditions? Cytosolic and mitochondrial-targeted geNOps would open the door to understanding subcellular NO dynamics from different sources such as cytosolic nitrate reductase (NR) or the mitochondrial electron transport chain. Probing for NO diffusion to the nucleus would allow deep insights into the role of NO in induction of hypoxia-responsive gene expression.

(2) What is the impact of stress intensity and duration on NO dynamics and signaling in the cell and what is the consequence on the stress response? Using a biosensor such as geNOps may facilitate monitoring spatio-temporal dynamics of NO in the early versus later stress stages as well as mild versus severe stresses.

(3) What is the spatio-temporal choreography of differential NO production between different regions of an organ, either during development or in response to stress? NO biosensing may allow a detailed insight into the organ-specific spatio-temporal distribution of NO.

(4) What is the organelle-specific NO dynamic during symbiosis? What are the respective contributions from different sources such as cytosolic NR, peroxisomal enzymes such as xanthine dehydrogenase (XDH), and the mitochondrial electron transport chain? Biosensing approaches may be able to map out patterns of organelle-specific NO dynamics during symbiosis.

However, geNOps has several limitations that need to be considered for developing its application particularly in planta (Eroglu et al., 2017): (1) geNOps function depends on heme which might impact measurements depending on the availability of endogenous heme inside the specific cellular location. (2) geNOps is pH sensitive. Acidic conditions cause decreased fluorescence of cyan and green geNOps. This is an important consideration as the cellular compartments vary in their pH particularly upon stress conditions such as hypoxia which decreases the cytosolic pH. Therefore, it is crucial to use a NO-insensitive control probe, geNOpsmut, for a meaningful interpretation of probe responses. (3) The intensional nature of the sensor makes it dependent on the expression level, and its fusion with a non-responsive normalizer fluorophore, such as mCherry, is recommended (Hung et al., 2011).

In conclusion, geNOps is a potential candidate for developing NO biosensing in planta. Future investigation should focus not only on finding the most suitable sensors for NO detection in plant cells but also on finding suitable fluorophores and tags for monitoring the subcellular localization (e.g., mitochondria, cytoplasm, plasma membrane, peroxi-some, plastid) of NO. Subcellular-targeted geNOps would

Figure 1 Different NO sources in plant cells and the basic structure of geNOps as potential FP sensors for plant NO bioimaging. Cytosolic NR, the key enzyme involved in nitrate assimilation which converts nitrate to nitrite, is the most famous enzyme documented to catalyze the conversion of nitrite to NO (Yamamoto-Katou et al., 2006). Besides NR, another MOCO enzyme, xanthine oxidoreductase (XOR) or XDH in plants, involved in purine catabolism in the peroxisome, synthesizes NO from nitrite in vitro (Godber et al., 2000). Moreover, cytochrome c oxidase, part of the electron transport chain (ETC) located in the inner membrane of mitochondria, is involved in NO synthesis from nitrite (Planchet et al., 2005). Under low oxygen condition, nitrite:NO reductase (NiNOR) activity, which has been discovered in the root plasma membrane (PM), uses the nitrite provided by PM bound NR as substrate (Stöhr et al., 2001). Under low pH, non-enzymatic conversion of nitrite to NO can occur in the apoplast and plastids (Bethke et al., 2004; Gas et al., 2009). The geNOps fluorescence decreases upon binding of NO to the Fe^2+ center, and this reaction is reversible in the absence or reduced level of NO (Eroglu et al., 2016).

### Table 1 Available genetically encoded FP sensors for NO detection

| Genetically encoded Probe name | Mechanism of function (based on NO or its derivatives) | Studied organism | Detection method | Reference |
|-------------------------------|------------------------------------------------------|------------------|----------------|----------|
| FRET-MT                       | Metallothionein-based NO reporter                     | Animal           | Fluorescence technology | Pearce et al. (2000) |
| NOA1                          | cGMP-based                                           | Animal           | Fluorescence technology | Sato et al. (2005) |
| pnGFP                         | Peroxy nitrite (ONOO^{-})-based                      | Animal           | Fluorescence technology | Chen et al. (2013) |
| sNOOPy                        | Nitrate and nitrite-based                            | Animal           | Fluorescence technology | Hidaka et al. (2016) |
| geNOps                        | NO-based                                             | Animal           | Fluorescence technology | Eroglu et al. (2016) |
| Lb^{2+} NO                    | Nitrosyl-plethoglobin-based                          | Plant            | Electron paramagnetic resonance spectroscopy (EPR) | Calvo-Begueria et al. (2018) |
enable monitoring NO spatio-temporal dynamics in real-time in planta.

Conflict of interest statement. The author declares no conflict of interest.

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