Nitric Oxide 25 (2011) 216–221

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Review

# Regulatory mechanisms of nitric oxide and reactive oxygen species generation and their role in plant immunity

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ARTICLE INFO

*Article history:*
Available online 30 December 2010

*Keywords:*
CDPK
NADPH oxidase
NO
MAPK
Riboflavin

ABSTRACT

Rapid production of nitric oxide (NO) and reactive oxygen species (ROS) has been implicated in diverse physiological processes, such as programmed cell death, development, cell elongation and hormonal signaling, in plants. Much attention has been paid to the regulation of plant innate immunity by these signal molecules. Recent studies provide evidence that an NADPH oxidase, respiratory burst oxidase homolog, is responsible for pathogen-responsive ROS burst. However, we still do not know about NO-producing enzymes, except for nitrate reductase, although many studies suggest the existence of NO synthase-like activity responsible for NO burst in plants. Here, we introduce regulatory mechanisms of NO and ROS bursts by mitogen-activated protein kinase cascades, calcium-dependent protein kinase or riboflavin and its derivatives, flavin mononucleotide and flavin adenine dinucleotide, and we discuss the roles of the bursts in defense responses against plant pathogens.



**Contents**



## Introduction

Timely recognition of invading microbes and rapid induction of defense responses are essential for plant disease resistance. At least two recognition systems are used by plants [1]. Plant basal defenses are often initiated by a much less specific recognition system that identifies microbe-associated molecular patterns (MAMPs) that are so-called general elicitors, such as flagellin [2] and lipopolysaccharides [3]. Both animals and plants can recognize invariant MAMPs that are characteristic of pathogenic microorganisms by pattern recognition receptors (PRRs) in the plasma membrane. Pathogens produce effectors to suppress defense, but plants, in turn, can sense such effectors by dominant plant resistance (*R*) gene products that are typically nucleotide-binding leucine-rich repeat proteins. Such intense active defense is known as gene-for-gene resistance. Plant PRRs and R proteins share similarities with components of the animal innate immune system, suggesting that some downstream signaling components are common between plants and animals [1].

Rapid production of nitric oxide (NO) and reactive oxygen species (ROS), called NO burst and ROS burst, respectively, have been implicated in diverse physiological processes, such as resistance to biotic and abiotic stress, hormonal signaling and development in plants [4–7]. Recently, NO has attracted attention as a radical that participates in plant innate immunity. NO activates the mitogen-activated protein kinase (MAPK) cascade [8] and increases expression of defense genes, such as those coding for phenylalanine ammonia-lyase and pathogenesis-related proteins [9]. In animals, NO is produced by NO synthase (NOS). The sources of NO synthesis in plants include reduction in nitrite by nitrate reductase, but oxidation of arginine to citrulline by NOS is still controversial. Although evidence for arginine-dependent NO synthesis in plants has accumulated, no gene or protein that has a sequence similar to known mammalian-type NOS has been found in plants

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doi:10.1016/j.niox.2010.12.008

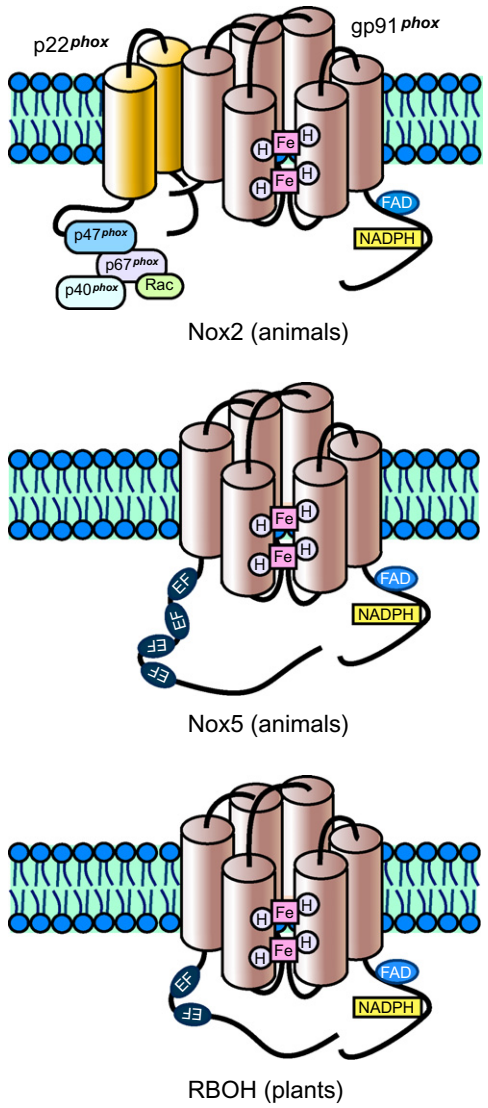


**Fig. 1.** Models for transmembrane topology and functional domains of animal Nox2, Nox5 and plant RBOH. Cylinders represent six transmembrane a-helices. EF, $Ca^{2+}$-binding EF-hand motif. Conserved histidines, heme groups, FAD and NADPH binding sites are indicated.

[10,11]. Guo et al. [12] identified a NOS-like enzyme (AtNOS1) from Arabidopsis (*Arabidopsis thaliana*) with a sequence similar to a protein that has been implicated in NO synthesis in the snail *Helix pomatia*. The AtNOS1 protein has no NOS activity [13], and therefore AtNOS1 was renamed AtNOA1 for NO ASSOCIATED1 [14]. Recent studies showed that AtNOA1 has circularly permuted GTPase activity in plastides [15]. However, Arabidopsis mutant *noa1* is still useful for its phenotype, which shows reduced levels of NO in plant growth, fertility, hormonal signaling, salt tolerance and defense responses [12,15–18]. Although association of AtNOA1 with NO seems to be simply the result of pleiotropic effects of malfunctioning plastides that overproduce ROS [15,19], knocking out or down *NOA1* provides one of the available tools to analyze NO function.

The activation mechanism of NADPH oxidase has been well investigated for mammals. NADPH oxidases have NADPH oxidase/dual oxidase (Nox/Duox) as a catalytic subunit. In phagocytes, $gp91^{phox}$, known as Nox2 (Fig. 1), forms a multi-protein complex with $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$ and Rac2 [20]. Nox organizer 1 (Noxo1) and Nox activator 1 (Noxa1) are homologs of $p47^{phox}$ and $p67^{phox}$, respectively, and are required for Nox1 activation [21]. Nox5, Duox1, and Duox2 have N-terminal extensions, including EF-hand motifs, and are likely regulated by $Ca^{2+}$ directly [20]. The respiratory burst oxidase homolog gene (*RBOH*) is a plant homolog of Nox5 (Fig. 1) in mammalian NADPH oxidase and is found in several plant genomes, such as Arabidopsis, rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), and *Nicotiana benthamiana* [22–29]. Several lines of evidence indicate that RBOH has a pivotal role in ROS-mediated signaling, such as defense responses, plant development and cell elongation. *AtRBOHD* and *AtRBOHF* function in ROS production during pathogen signals [30] and abscisic acid-induced stomatal closure in guard cells [31]. Virus-induced gene silencing (VIGS) of *NbRBOHA* and *NbRBOHB* in *N. benthamiana* attenuates ROS production and resistance to *Phytophthora infestans* [27,32]. Loss of function of tomato *RBOHs* by an antisense technique reduces ROS production in leaves and induces morphological abnormality [33]. In root hair development, ROS production by *AtRBOHC/RHD2* controls cell expansion through activation of $Ca^{2+}$ channels [34]. Except for RBOH and Rac, the homologs of other subunits of phagocyte NADPH oxidase have not been found by Arabidopsis genome sequencing [35]. RBOH protein localizes on the plasma membrane [36] (Fig. 1). The N-terminal extension includes two $Ca^{2+}$-binding EF-hand motifs and suggests participation of $Ca^{2+}$ signaling in the activation process [23]. Sagi and Fluhr [37] showed that $Ca^{2+}$ directly activates an RBOH-like enzyme in tomato and tobacco plasma membranes by using denaturing gel assay and then by regeneration. Rac GTPase is also implicated to regulate RBOH by means of N-terminal extension. Wong et al. [38] indicated direct interaction between Rac and the N-terminal of RBOH that may activate NADPH oxidase activity in plants. They also suggested that cytosolic $Ca^{2+}$ concentration might modulate NADPH oxidase activity by regulating interaction between Rac and RBOH. A calcium-dependent protein kinase (CDPK) activates NADPH oxidase by direct phosphorylation of its N-terminal region [39]. Other studies also suggest the participation of interactors in the process of RBOH activation: activation of rice RBOHs was linked to the expression of RACK1 (an interactor with Rac1) [40], and tobacco cells transformed with an antisense construct of 14-3-3 protein, which interacts with the C-terminal extension of NtRBOHD, reduced accumulation of ROS after cryptogein treatment [41]. Phosphatidic acid binding to the N-terminus of Arabidopsis AtRBOHD and AtRBOHF results in activation of NADPH oxidase and ROS production in guard cells [42].

NO and ROS together, but not individually, are required to induce cell death [43], and balanced production of NO and $H_2O_2$ is important to induce hypersensitive response (HR) cell death [44]. However, little is known how these molecules are coordinately regulated and their functions in plant immune responses. Recently, we found that MAPKs regulate production of NOA1-associated NO and NADPH oxidase-dependent ROS [32]. In this review, we discuss the molecular mechanisms of the regulation of NO and ROS production and the roles of radical burst in resistance to pathogens.

## CDPK activates NADPH oxidase by direct phosphorylation

Transient influx of $Ca^{2+}$ into the cytoplasm after recognition of pathogen signals is an early stage of signaling cascades that trigger ROS burst and HR cell death [45–47]. CDPKs are Ser/Thr protein kinases that include a $Ca^{2+}$-binding calmodulin-like domain and are the best characterized calcium sensors in plants. CDPKs are encoded by a large multigene family and have possible redundancy or diversity or both in their functions [48,49]. Accumulating evidence indicates that CDPKs regulate many aspects of plant growth and development [50,51], hormonal responses [52–54] and adap-