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Research review paper

Versatile roles of plant NADPH oxidases and emerging concepts

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ABSTRACT

NADPH oxidase (NOX) is a key player in the network of reactive oxygen species (ROS) producing enzymes. It catalyzes the production of superoxide (O_2^-), that in turn regulates a wide range of biological functions in a broad range of organisms. Plant Noxes are known as respiratory burst oxidase homologs (Rbohs) and are homologs of catalytic subunit of mammalian phagocyte gp91^{phox}. They are unique among other ROS producing mechanisms in plants as they integrate different signal transduction pathways in plants. In recent years, there has been addition of knowledge on various aspects related to its structure, regulatory components and associated mechanisms, and its plethora of biological functions. This update highlights some of the recent developments in the field with particular reference to important members of the plant kingdom.

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Introduction

Reactive oxygen species (ROS) plays a central role in regulating various biological functions in living organisms. The intracellular balance of ROS is tightly regulated by ROS producing and scavenging systems. Although, there are various sources of ROS generation in plants, NADPH oxidase (NOX) is the most extensively studied. NADPH oxidase is an enzyme complex, localized on the plasma membrane, utilizes NADPH as an electron donor and catalyzes the production of apoplastic superoxide radicals (O_2^-). Since its initial role in activating blood phagocytic cells in human and thereby killing pathogens, the research in this area has gained a lot of momentum. At present, NADPH oxidase enzyme is found to be widely distributed in a broad range of organisms including vertebrates, invertebrates, higher plants and fungi. In plants, NADPH oxidases are known as respiratory burst oxidase homologs (Rbohs)

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and constitute a multigene family (Sagi and Fluhr, 2006). In plants, a number of Rbohs have been identified which spread across 27 plant species including 19 dicots, 5 monocots and 3 lower plants of known as well as unknown functions as described in Table 1. Among various ROS producing enzyme systems in plants, Rbohs occupy a unique position due to their integration into different signaling pathways. Various post-translational modifications have also resulted into their localized and systemic signal transduction. With the availability of biological resources and development of precise scientific tools, there has been rapid advancement of knowledge in the area, which has been discussed by several reviewers (Baxter et al., 2013; Mittler et al., 2011; Suzuki et al., 2011, 2012). We review some of the recent developments in terms of their structure, identification of regulatory components involved in signal transduction mechanisms and their key biological functions in order to understand the roles of NADPH oxidase in plant growth, development and in stress responses.

Structure of plant NADPH oxidase

The NADPH oxidase, first identified in human phagocytic cells, comprised of a membrane-bound flavocytochrome b_{558}, three cytosolic subunits ($p47^{phox}$, $p67^{phox}$ and $p40^{phox}$) and a small GTPase Rac1 (macrophages) or Rac2 (neutrophils). The flavocytochrome b_{558} is further composed of two components: a flavin and heme-binding glycoprotein of 91 kDa (called as $gp91^{phox}$ or Nox2); and a small subunit of 21 kDa (called $p22^{phox}$). Phosphorylation of cytosolic subunits plays a critical role in phox activation (Bedard and Krause, 2007). The first plant homolog of mammalian $gp91^{phox}$ was identified in rice known as *OsRbohA* (*Oryza sativa* respiratory burst oxidase homolog A; Groom et al., 1996). Subsequently, more *Rbohs* were identified in many other plant species including dicots, monocots and lower plants. Unlike animals, plant Nox(s) possess two major structural components, namely respiratory burst oxidase homolog (Rboh; 105–112 kDa homolog of $gp91^{phox}$) and its cytosolic regulator Rop (Rho-like protein; a Rac homolog of plants) (Yoshie et al., 2005). Similar to animals, the C-terminal end of Rbohs has NADPH and FAD-binding domains; and six trans-membrane domains (TMD-1 to TMD-6) connected by five loops (loops A–E), where TMD-3 and TMD-5 contain pairs of His residues required for heme-binding. Apart from these domains, amino acid residues essential for its catalytic activity (Pro-415 and Asp-500 of Nox2), are also conserved (Kawahara et al., 2007). The recently solved structure of N-terminal region (138–313 amino acid residues) of *O. sativa* RbohB ($OsRbohB^{138–313}$) at 2.4 Å resolution and its proposed mode of dimerization (Oda et al., 2008) have provided a missing link for previous biochemical and functional studies. Structural elucidation further suggests the presence of two EF-hands (EF-1 and EF-2) on hydrophilic N-terminal (approximately 300 residues) extension of Rbohs, which may act as a bridge between Rboh and Rop, working as a substitute to the mammalian $p67^{phox}$ homolog (Wong et al., 2007). Each chain of homodimer is composed of eight α-helices, two β-strands (Fig. 1A and B) and two EF-hands (each with helix–loop–helix secondary structure as well as the ligands presented by the loop to bind the Ca^{2+} ion; Fig. 1C) with Ca^{2+}-binding sites viz. Asp242, Asn244, Asp246, Arg-248 and Glu253 in EF-hand I (Fig. 1D). Swapping EF-hands in the homodimer contributes to its stabilization as well as create a binding region for Rop (OsRac1) within the coiled-coil region (Fig. 1E). Further, Ca^{2+}-dependent conformational change by binding of calcium to EF-hand and Ca^{2+}-independent intramolecular interaction between N- and C-terminus have also been reported. Two additional EF-hand-like motifs (EF-like 1 and EF-like 2) from the structural analysis have also been identified (Fig. 1F). The domain formed by EF-hand-like motifs maintains the structural characteristics of EF-hand 1 (Oda et al., 2010). Recently, an attempt was made to purify and crystallize the Rac/Rop GTPase of rice (OsRac1) at 1.9 Å resolution to get insights into the activation of GTPase, an important regulatory switch (Kosami et al., 2014). GTP analog 5′-guanylyl imidodiphosphate (GMPPNP) was used for co-crystallization experiment and the crystal belonged to space group $P2_12_12_1$ was obtained.

Regulatory components of NADPH oxidase and its activation

Because of structural difference with animal Noxes, activation and regulation of Rbohs are different from those of animals. The activity of NADPH oxidase involves a systematic and coordinated interaction of different components. The principal regulatory components involve Ca^{2+} (Ogasawara et al., 2008), calcium-dependent protein kinases (CDPKs are Ser/Thr protein kinases that include a Ca^{2+}-binding calmodulin-like domain) (Kobayashi et al., 2007), Ca^{2+}/CaM-dependent protein kinase (CCaMK) (Shi et al., 2012) and Rop (Wong et al., 2007). However, the association of other regulatory components such as extracellular ATP (eATP), Phospholipase Dα1 (PLD α1) and its lipid product phosphatidic acid (PA; a lipid signaling molecule derived from ABA-activated PLDα1), mitogen activated protein kinase (MAPK), a member of 14–3–3 protein family (Nt14–3–3h/omega1) and nitric oxide (NO) has also been demonstrated. The present review provides an overview of the association of various components towards Rboh activation.

Role of calcium and protein kinases

Out of the proposed 7 Nox subfamilies viz. Nox1–3, Nox4, Nox5, NoxA/B, NoxC/D, Duox and plant Nox (Rbohs), 4 are calcium-regulated (Kawahara et al., 2007). Elicitor/pathogen induced cytosolic Ca^{2+} influx activates AtRbohD by dual mechanisms synergistically: directly by conformational change in EF-hand region, and indirectly via phosphorylation of the N-terminal through CDPKs (Ogasawara et al., 2008). A recent work demonstrated the regulation of *Arabidopsis* NADPH oxidase (AtRbohF) by phosphorylation of the N-terminal domain via the function of CBL1/CBL9–CIPK26 (Calcineurin B-Like calcium sensors–interacting protein kinase) complex and by direct interaction of Ca^{2+} to the EF-hand motifs (Drerup et al., 2013). Seven different residues in AtRbohD undergo phosphorylation, whereas phosphorylation at $Ser^{343/347}$ was critical for activation of AtRbohD (Benschop et al., 2007; Nühse et al., 2007). Kobayashi et al. (2007) demonstrated that StCDPK5 (a potato CDPK) activates StRbohA–D by direct phosphorylation of the N-terminal region. Ser-82 and Ser-97 were identified as potential phosphorylation sites at the N-terminus of StRbohB. Until recently, the substrate specificity of CDPKs involved in diverse physiological processes was largely unknown. Recently, for the first time, the contribution of variable (V) domain of StCDPK5 for localization and substrate specificity of CDPKs in vivo has been demonstrated (Asai et al., 2013). The experiment conducted by substitution of the V domain of StCDPK5 with tomato SlCDPK2 resulted in impairment of phosphorylation abilities of StRbohB. Further, the cooperative effort of Ca^{2+} and phosphorylation in the activation of OsRbohB has been proposed. The role of the upstream N-terminal region in Ca^{2+}- dependent activation but not in phosphorylation-induced process was also observed in OsRbohB (Takahashi et al., 2012). It has also been demonstrated that Ca^{2+}-binding to EF-hands is required for the activation of AtRbohC (Takeda et al., 2008). A recent study revealed that rice Ca^{2+}/CaM-dependent protein kinase (CCaMK) OsDMI3 regulates the expression of NADPH oxidase genes, OsRbohB, OsRbohE, and OsRbohI, and the production of H_2O_2 in ABA signaling (Shi et al., 2012). In addition to CDPKs, ABA-activated SnRK2 protein kinase open stomata 1 (OST1) (SRK2E/SnRK2.6) was also involved in the physical interaction with *Arabidopsis* Rboh (AtRbohF) via phosphorylation at Ser13 and Ser174 residues, regulating its activity during stomatal closure (Sirichandra et al., 2009). Another kinase, calcineurin B-like protein (CBL)-interacting protein kinase 26 (CIPK26) represses the activity of AtRbohF, either by conformational change on the N-terminal region or by phosphorylation (Kimura et al., 2012).