Signaling and Communication in Plants

Series Editors

František Baluška
Department of Plant Cell Biology, IZMB, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

Jorge Vivanco
Center for Rhizosphere Biology, Colorado State University, 217 Shepardson Building, Fort Collins, CO 80523-1173, USA

Luis Alfonso del Río • Alain Puppo
Editors

Reactive Oxygen Species in Plant Signaling

Springer

Editors
Prof. Luis A. del Río
Estación Experimental del
Zaidín
CSIC
Depto. Bioquímica
Biología Celular y
Molecular de Plantas
Profesor Albareda, 1
E-18008 Granada
Spain

Prof. Alain Puppo
Interactions Biotiques et Santé Végétale
UMR INRA 1301/Université de Nice-Sophia
Antipolis/CNRS 6243
400, route des Chappes, BP 167
06903 Sophia Antipolis Cedex
France

ISBN: 978-3-642-00389-9 e-ISBN: 978-3-642-00390-5
DOI: 10.1007/978-3-642-00390-5
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009921146

Cover design: WMXDesign GmbH, Heidelberg, Germany

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Oxygen (O_2) appeared in significant amounts in the Earth's atmosphere over 2.2 billion years ago, largely due to the evolution of photosynthesis by cyanobacteria (Halliwell 2006). The O_2 molecule is a free radical, as it has two impaired electrons that have the same spin quantum number. This spin restriction makes O_2 prefer to accept its electrons one at a time, leading to the generation of the so-called reactive oxygen species (ROS). The chemical nature of these species dictates that they can create damage in cells. This has contributed to the creation of the "oxidative stress" concept; in this view, ROS are unavoidable toxic products of O_2 metabolism and aerobic organisms have evolved antioxidant defences to protect against this toxicity (Halliwell 1981; Fridovich 1998). Indeed, even in present-day plants, which are full of antioxidants, much of the protein synthetic activity of chloroplasts is used to replace oxidatively damaged D1 and other proteins (Halliwell 2006). Yet, the use of the "oxidative stress" term implies that ROS exert their effects through indiscriminate widespread inactivation of cellular functions. In this context, ROS must not be able to react with lipids, proteins or nucleic acids in order to avoid any damage to vital cellular components.

However, genetic evidence has suggested that, in planta, purely physicochemical damage may be more limited than previously thought (Foyer and Noctor 2005). Thus, the concept of "oxidative stress", which implies a state to be avoided, was re-evaluated and the term "oxidative signaling" was created (Foyer and Noctor 2005). This means that ROS production, which was originally considered as an exclusively harmful process, is also an important component of the signaling network that plants use for their development and for responding to environmental challenges. The evolution of efficient antioxidant systems has most likely enabled plant cells to overcome ROS toxicity and to use these reactive species as signal transducers (Mittler 2006).

Results obtained during the last decade have highlighted the role of ROS as signals in plants, and it is now widely accepted that ROS are key regulators of plant metabolism, morphology, and development. The role of ROS as signals for gene expression has been evidenced (Desikan et al. 2001; Vanderauwera et al. 2005), and it is now known that ROS modulate the activity of key signaling compounds such as MAP kinases (Rentel et al. 2004). Furthermore, ROS can induce protein modifications, and thiol changes have been suggested as a widespread mechanism by which ROS might affect the activity and function of proteins (Cooper et al. 2002).

The identification of ROS-generating enzymes has demonstrated that plant cells can initiate ROS production for the purpose of signaling, and that the spatio-temporal characteristics of this production are likely to play an important role in the transduction of ROS signals (Mittler 2006). It is now obvious that sophisticated processes regulate these characteristics, which lead to the generation of inter-compartmental gradients, at least of hydrogen peroxide (H_2O_2). Transport of H_2O_2 through vesicle trafficking (Leshem et al. 2006) is most probably an important component of this process. Moreover, the facilitated diffusion of H_2O_2 across membranes through specific aquaporins (Bienert et al. 2007) appears to play a crucial role in the establishment of such H_2O_2 gradients. On the other hand, it appears that oxidation of target molecules by ROS is a part of how plants perceive and respond to environmental and developmental triggers (Foyer and Noctor 2005). Among the processes where ROS involvement has been evidenced, one can first cite the plant pathogen defense. During this process, ROS are produced by plant cells via plasma membrane-bound NADPH-oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast (Bolwell 1999; Grant and Loake 2000). ROS production leads to programmed cell death (Bolwell 1999) and orchestrates the plant hypersensitive disease resistance response (Levine et al. 1994). Moreover, it has been shown that H_2O_2 can mediate the systemic expression of defense-related genes (Orozco-Cardenas et al. 2001). ROS appear to also play an important role in plant development and functioning. They are essential signals in stomatal closure in response to abscisic acid and elicitors (Pei et al. 2000). They are involved in root hair growth (Foreman et al. 2003) and auxin signaling and gravitropism (Joo et al. 2001). ROS induce the expression of genes encoding proteins required for peroxisome biogenesis (López-Huertas et al. 2000). Moreover, it has recently been shown that DELLAs proteins regulate plant growth and defense processes by modulating the levels of ROS (Achard et al. 2008).

The volume of research into the roles of ROS in plants is currently growing. Thus, the purpose of this book is to present recent advances in this field. The constitutive chapters are mainly arranged around four topics: (1) the generation of ROS, their network signalling, including the retrograde signalling from the chloroplast to the nucleus and the cross-talk with hormone signaling, (2) the signaling role of ROS produced in some sub-cellular compartments, (3) the role of ROS in plant growth, development, functioning and stress acclimation, and (4) their role in biotic and abiotic interactions. In thanking the authors for their contributions, we are convinced that the forthcoming years will bring new exciting insights into this field. Unravelling the sensing and transduction of ROS, including the post-translational regulation, the modulation of their concentration at subcellular level, and the interaction networks with the reactive nitrogen species, as well as their possible role in epigenetic processes, will shed new light on ROS action in plants.

Granada, April 2009 Luis A. del Río
Sophia-Antipolis, April 2009 Alain Puppo

References

Achard P, Renou JP, Berthome R, Harberd NP, Genschik P (2008) Plant DELLAs restrain growth and promote survival of adversity by reducing the levels of reactive oxygen species. Curr Biol 18: 656–660.

Bienert GP, Møller ALB, Kristiansen KA, Schultz A, Møller IM, Schjoerring JK, Jahn TP (2007) Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem 282: 1183–1192.

Bolwell GP (1999) Role of active oxygen species and NO in plant defense responses. Curr Opin Plant Biol 2: 287–294.

Cooper CE, Patel RP, Brookes PS, Darley-Usmar VM (2002) Nanotransducers in cellular redox signalling: modification of thiols by reactive oxygen and nitrogen species. Trends Biochem Sci 27: 489–492.

Desikan R, A-H-Mackerness S, Hancock JT, Neill SJ (2001) Regulation of the Arabidopsis transcriptome by oxidative stress. Plant Physiol 127: 159–172.

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 27: 442–446.

Foyer CH, Noctor G (2005) Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ 28: 1056–1071.

Fridovich I (1998) Oxygen toxicity: a radical explanation. J Exp Biol 201: 1203–1209.

Grant JJ, Loake GJ (2000) Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol 124: 21–29.

Halliwell B (1981) Toxic effects of oxygen on plant tissues. In: Chloroplast metabolism: the structure and function of chloroplasts in green leaf cells, Clarenden Press, Oxford.

Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141: 312–322.

Joo JH, Bae YS, Lee JS (2001) Role of auxin-induced reactive oxygen species in root gravitropism. Plant Physiol 126: 1055–1060.

Leshem Y, Melamed-Book N, Cagnac O, Ronen G, Nishri Y, Solomon M, Cohen G, Levine A (2006) Suppression of Arabidopsis vesicle-SNARE expression inhibited fusion of H_2O_2-containing vesicles with tonoplast and increased salt tolerance. Proc. Natl. Acad. Sci. USA 103: 18008–18013.

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583–593.

López-Huertas E, Charlton WL, Johnson B, Graham IA, Baker A (2000) Stress induces peroxisome biogenesis genes. EMBO J 19: 6770–6777.

Mittler R (2006) The roles of reactive oxygen species in plant cells. Plant Physiol 141: 311.

Orozco-Cardenas ML, Narvaez-Vasquez J, Ryan CA (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell 13: 179–191.

Pei Z-M, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. Nature 406: 731–734.

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Peterson L, Okamoto H, Knight H, Peck SC, Grierson CS, Hirt H, Knight MR (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. Nature 427: 858–861.

Vanderauwera S, Zimmermann P, Rombauts S, Vandenabeele S, Langebartels C, Gruissem W, Inze D, Van Breusegem F (2005) Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiol 139: 806–821.

Contents

Reactive Oxygen-Generating NADPH Oxidases in Plants

Robert Fluhr

Abstract Reactive oxygens produced by the integral membrane protein NADPH oxidase (NOX) function in defense, development, and redox-dependent signaling. They share common structural features and are evolutionarily of ancient origin and thus ubiquitous in eukaryotes. In plants, NOX are part of a multigene family and are implicated in diverse events including innate immunity and development. Due to the fact that reactive oxygens are toxic, and in many cases short-lived, the activity of these oxidases is tightly regulated both temporally and spatially. The recent elucidation of domains for activation by calcium and Rac binding in NOX as well as its positioning on membrane lipid rafts sketches a fascinating picture of its functional dynamics. This review draws upon comparative structure–function relationships between plant and animal NOXs to portray novel aspects of their biology.

1 Diversity of the NADPH Oxidase Superfamily Basic Structure and Evolutionary Aspects

Reactive oxygen species (ROS) produced by NADPH oxidase (NOX) have been shown to play many critical roles in signaling and development in plants, including plant defense response, cell death, abiotic stress, stomatal closure, and root hair development (Foreman et al. 2003; Jones et al. 2007; Kwak et al. 2003; Torres et al. 2002; Yoshioka et al. 2003). Yet ROS species can be cytotoxic and mutagenic and for their proper function in signaling their production must be carefully controlled. The NOXs are part of a large superfamily that arose early in evolution before animal/plant divergence. Plant NOXs are called RBOH (respiratory burst oxidase homologs). *Arabidopsis* contains ten homologues that comprise a distinct phylogenetic clade

R. Fluhr
Department of Plant Sciences, Weizmann Institute of Science, P.O.B. 26, Rehovot, 76100 Israel
e-mail: robert.fluhr@weizmann.ac.il

L.A. del Río and A. Puppo (eds.), *Reactive Oxygen Species in Plant Signaling*, Signaling and Communication in Plants, DOI 10.1007/978-3-642-00390-5_1,

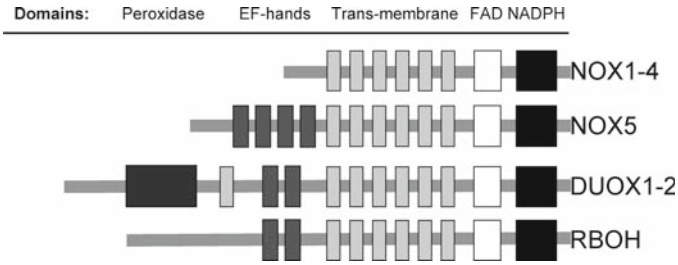

Fig. 1 Select family members of the NADPH oxidase (NOX) superfamily. The NOX/RBOH domain contains six transmembrane helices that are found in the core NOX2 structure and are found in all members of the NOX family. The NOX1-5 and DUOX1,2 are human proteins where NOX5 contains EF-hands and DUOX1,2 an additional peroxidase domain. Plant RBOH NOX homologues contain the NOX2 core and two additional EF hands

among the NOX-related genes (Sagi and Fluhr 2006). Plant RBOH are related in a structural sense to human NOX (Fig. 1) which presents a convenient paradigm for developing our understanding of RBOH biology.

In humans, there are five different NOX structural types. NOX1–3 contains the basic NOX domain and needs interaction with accessory proteins to be fully active. For example, NOX2, one of the best studied oxidases of this family, is prominent in phagocyte biology forming a complex called phox (gp91^{phox}, phagocyte NOX). It requires p22^{phox} to form the flavocytochrome heterodimer b_{558} and additional regulatory subunits p67^{phox}, p47^{phox}, p40^{phox}, and the small GTPase Rac (Bedard and Krause 2007; Lambeth 2004). In contrast, the closely related NOX4 associates in epithelial cells with p22^{phox} to produce large amounts of ROS constitutively and does not require accessory subunits or GTPase Rac for this activity (Martyn et al. 2006). Other structurally diverse human forms are NOX5 and DUOX1 and DUOX2 (Fig. 1). NOX5 has a N-terminal extension with EF-hand containing calcium-binding domains, while the DUOX members are characterized by a N-terminal peroxidase domain (Banfi et al. 2001; Edens et al. 2001).

Interestingly, not all animal species contain the full NOX complement found in humans, for example rat and mouse lack NOX5 having apparently lost it during their evolution. Simpler eukaryotes like yeast completely lack NOX homologues (Kawahara et al. 2007). At least three classes of NOX homologues, in a highly divergent form, are prevalent in fungi where they are thought to participate in sexual development, cellular differentiation, and appressorial formation (Aguirre et al. 2005; Giesbert et al. 2008; Takemoto et al. 2007) or play a role in fungal–host pathogenicity (Giesbert et al. 2008; Segmuller et al. 2008). Specialized NOX types continue to evolve in animals. NOX3, structurally related to NOX2, is a recent NOX derivative found only in mammals and birds. It is localized to the vestibular and cochlear sensory epithelia and spiral ganglions (Banfi et al. 2004a) apparently necessary for the formation of mineralized structures that are important for gravity perception in those organisms (Paffenholz et al. 2004).

2 RBOH Multi Gene Family

The presence of large gene families in organisms is usually an indication that each gene has specificity in its function together with a varying degree of functional overlap. Inspection of digital northern activities in *Arabidopsis* gathered from recent Affymetrix microarray slides reflects a high level of gene specialization (Table 1) (Zimmermann et al. 2004, 2005) The tissue-specific division of RBOH transcript distribution falls into three basic classes; expression throughout the plant (AtrbohD and F), in the roots (Atrboh A-G, I), and in a pollen-specific manner (AtrbohH and J). The tissue-specific expression is reflected in the phylogenetic distribution in which H and J form a small sub-clade (Sagi and Fluhr 2006). The most common abiotic inducers of Atrboh transcript accumulation include conditions

Table 1 Tissue specificity and response activities of atrboh transcripts

RBOH	AGI	Localization[a]	Induction characteristics[b]
A	At5g07390	Root, elongation zone	Hypoxia/salt stress, genotoxic, nitrogen starvation, fad2 mutant. Repressed: in *oxt6* (redox gene regulator)
B	At1g09090	Root, elongation zone	Anoxia, hypoxia, methyl jasmonate, UV-B, elevated in rbohC mutant. Repressed: ABA, cold, zeatin cycloheximide, myb 50, leaf1 mutant, repressed by miR164b, *oxt6*
C	At5g51060	Root, elongation zone	Most highly expressed transcript of the RBOH class. *B. cinerea, P. syringae*: *Agrobacterium*, ozone. Repressed: cycloheximide, H_2O_2, 6-benzyl adenine
D	At5g47910	All plant parts, less in roots	Cycloheximide, anoxia, H_2O_2, chitin, ozone, $AgNO_3$, methyl jasmonate, *max4* (inhibits shoot branching), *F. occidentalis, P. infestens, P, syringae*. Repressed: ABA, high CO_2, fls2-17
E	At1g19230	Cell suspension, root, seeds	*Agrobacterium*, nitrogen starvation,, miR156b genotoxic. Repressed: senescence, miR172a, *oxt6*
F	At1g64060	All plant parts	*Agrobacterium*, brassinolide. Repressed: isoxaben, *mpk4* (constitutive SAR)
G	At4g25090	Root, elongation zone	Low nitrogen, salicylic acid, glucose sucrose miR172a. Repressed: myb61.2
H	At5g60010	Stamens, pollen	Repressed *oxt6*
I	At4g11230	Roots, elongation zone	Anoxia, cycloheximide, norflurazone, HSP90. Repressed: sfr6.4, *oxt6*
J	At3g45810	Stamens, pollen	Upregulated in rhd2 background, sfr6.1. Repressed: in camta1-2, *oxt6*

[a] Based on 3,110 microarray database compiled in Genevestigator V3. Results shown are significantly higher than background ($p \leq 0.06$). Experiments are summarized in https://www.genevestigator.ethz.ch. (Zimmermann et al. 2005)

[b] Induction of more than twofold or where indicated repressed by 1/2-fold and above 200 in the relative signal value.

of anoxia/hypoxia and nitrogen stress where AtrbohC and F are also induced by a variety of biotic stress. AtrbohC was specifically identified as playing a role in root hair development (Foreman et al. 2003), while AtrbohD is the major constitutively active form and AtrbohF is a biotic stress induced form (Torres et al. 2002). Both AtrbohD and F contribute to innate immune defense and ABA dependent guard cell opening, but only AtrbohF appears to be important in ABA-dependent root shortening (Kwak et al. 2003; Torres et al. 2002). Inspection of the transcriptional behavior of the RBOH family shows that these genes are in some cases regulated in concert. Thus, many of the RBOH family in *Arabidopsis* (6/10, Table 1) are suppressed in the *oxt6* mutant background (oxidative stress tolerant) that displays reduced sensitivity to a catalase inhibitor 3-amino-1, 2, 4-triazole (AT) and buthionine S,R-sulfoximine (BSO), an inhibitor of glutathione synthesis. The *oxt6* mutation involves inactivation of the specificity factor in the processing of transcript cleavage and polyadenylation (Zhang et al. 2008). Transcriptional profiling of oxt6 mutants detected elevated constitutive upregulated expression of a subset of genes that encode proteins containing thioredoxin- and glutaredoxin-related domains that represent alternative ROS-scavenging pathways. Thus, it is of note that oxt6 represses, in a reciprocal manner, members of the RBOH family, i.e., that are potential sources of ROS, and points to a common control mechanism for those genes.

3 Structurally Conserved Aspects of NOXs

Membrane proteins are notoriously difficult to crystallize and so far no crystal-based structure of a NOX is available. Thus, our functional understanding of its structure is based on decoding sequence motifs, analysis of the effects of mutations, and biochemical studies. The core NOX-like domain is characterized by six transmembrane helices that support two heme groups bound to conserved histidines as shown in Fig. 2. The C-terminal contains topologically distinct hydrophilic domains for NADPH and FAD. The NADPH interaction sites have been identified in human NOX2 by competition of the photoaffinity ligand 2 azido-NADPH by NADPH (Segal et al. 1992). The amino acid sequences have further been identified by direct comparison to the NADPH-binding domains of spinach ferrodoxin NADPH reductase as established by crystal structure (Rotrosen et al. 1992). A comparison of these motifs in relation to AtrbohD sequence is shown in Fig. 3.

Other important domains can be discerned by identifying those mutations in human NOX2 that confer dysfunction and are also conserved in the RBOH sequence. The classic examples have been gleaned from mutations that cause chronic granulomatous disease (CGD), a genetic immune disorder caused by point mutations in NOX2. People with CGD are susceptible to ingress of exotic microorganism not usually encountered in people with normal immune systems, e.g., *Aspergillus* growth. The recurrent infections can cause tumor-like masses called granulomas. Some of the point mutations are conserved throughout all NOX types and cause instability of the polypeptide or its inactivity. In this manner, localization of the

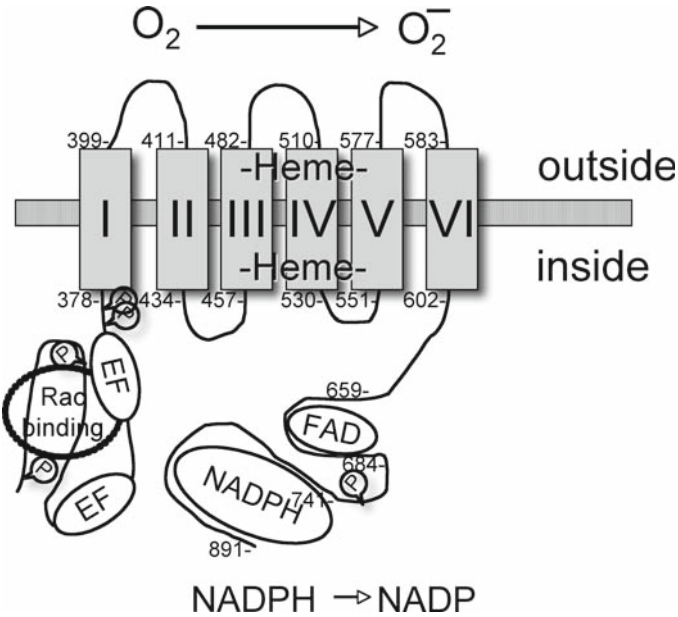

Fig. 2 Diagram of AtrbohD membrane localization. The conversion of NADPH to NADP will result in vectoral transfer of electrons with the participation of FAD and heme to an oxygen acceptor yielding superoxides. The core NOX-like domain is characterized by six transmembrane helices labeled I-VI. The two heme groups bound to conserved histidines that are present in helices III and V. The predicted "cytoplasmic side" contains binding cavities for FAD and the NADPH substrate. The presence of EF-hands and a putative Rac ineteracting site are depicted in the N-terminal region. Putative amino acid locations for transmembrane spanning approximations are based on the inspection of TMpred – Prediction of Transmembrane Regions and Orientation (http://www.ch.embnet.org/software/TMPRED_form.html) of the AtrbohD sequence and were further modified by manual alignment comparison to NOX2 and NOX5 sequence (Kawahara et al. 2007). *P* indicates putative phosphorylated amino acids and *Rac* indicates Rac/Rop small GTPases putative binding as discussed in the text

FAD binding region was predicted from studies using plasma membranes from a CGD patient with a point mutation at His-338. In that case, the NOX2 showed low FAD content in the plasma membrane and failed to produce superoxide (Yoshida et al. 1998). His-338 is located in the conserved amino acid motif ^{338}HPFT of human NOX2 which differs from the region suggested previously (Rotrosen et al. 1992). The equivalent conserved motif in AtrbohD is ^{660}HPFS. In human gp91^{phox}, mutants that are unable to reduce oxygen still retain diaphorase activity as measured by the reduction of iodonitrotetrazolium violet by NADPH (Cross et al. 1995).

Electrons from reduced FAD are transferred to two asymmetrical hemes that are ligated to histidine residues found in the transmembrane domains III and V. The exact positions of the histidines were established by alignment of gp91^{phox} to the yeast iron reductase. Yeast and plant iron reductases function to reduce exogenous ferric ions before their uptake. Both membrane proteins show a great deal of homology to

Gene	NADP pyrophoshate		NADP ribose		Adenine ring		C-4 atom of Nicotinamide
FNR	^{167}MLGTGTGIAPF	23	^{201}FLGVP	28	^{234}SREQTNEKGEKMYIQ	22	^{270}YMCG
NOX2	^{406}LVGAGIGVTPF	25	^{442}YWLCR	57	^{504}GLKQ----KTLYGR	21	^{535}FLCG
AtrbohD	^{741}LVGLGIGATPM	29	^{780}YWVT	72	^{856}GTRVK–SHFAKP	32	^{888}FYCG

Fig. 3 Spacing of NADPH interacting domains in AtrbohD compared to NOX2 and spinach ferrodoxin NADPH reductase (FNR) as defined by crystallography of FNR. The amino acid position is indicated at the start of each subdomain. The number of amino acids between each subdomain is indicated

other animal NOXs. The histidines in the yeast iron reductase were sequentially mutated and the resultant heme spectral activity monitored to reveal which mutations disrupted heme ligation capability (Finegold et al. 1996). Based on this work, the conserved AtrbohD histidines that serve as ligands for the hemes can be identified as follows: the cytoplasmic-proximal heme is ligated to histidines H459 and H561 and the outer heme ligation is to histidine H473 and H574 (Fig. 2). Incorporation of heme is also an important checkpoint in NOX biosynthesis. Indeed, blocking heme synthesis by application of succinyl acetone completely inhibited heterodimer formation of p22phox and gp65 which together form the core human NOX (DeLeo et al. 2000). Thus, it appears that insertion of the heme group is necessary for stabilization of the gp65 precomplex. The animal NOX2 oxidases are highly glycosylated indicating their passage through the Golgi, although the inhibition of glycolysation by application of tunicamycin does not affect the formation of stable heterodimer (DeLeo et al. 2000).

Additional domains of importance for the function of NOX2 that are highly conserved include: H209R, a conserved histidine binding residue (AtrbohD, 473); G389E, situated between the FAD and NADPH domains (AtrbohD, G713); and L420P (AtrbohD, L755) and W516R (AtrbohD, 869) that are in the NADPH domain. In comparison to the human NOX2, the NOX5 and RBOH proteins have shorter A and E extracellular loops (between transmembrane domains I and II and V and VI, respectively), as well as variable insertions between the FAD and NADPH domains (Fig. 2) (Kawahara et al. 2007). These conserved structural features are invaluable orientation guides that facilitate the transfer of structure-function relationships within the NOX superfamily.

4 Chemical Highlights of Superoxide Formation

Our knowledge of plant NOX chemistry is garnered from studies conducted in the animal NOX family (Bedard and Krause 2007; Vignais 2002). NOXs are transmembrane redox chain proteins that connect the electron donor, nicotinamide adenine dinucleotide phosphate (NADPH), on the cytosolic side of the membrane with the electron acceptor, oxygen, on the outer side of the membrane (Fig. 2). NADPH transfers two electrons (1.1) that culminates in the reduction of two oxygen molecules (1.2).

$$\mathrm{NADPH} - > \mathrm{NADP}^{+} + \mathrm{H}^{+} + 2\mathrm{e}^{-} \tag{1.1}$$

$$\mathrm{NADPH} + 2\mathrm{O}_2 - > \mathrm{NADP}^{+} + 2\mathrm{O}_2^{-} + \mathrm{H}^{+} \tag{1.2}$$

In the first step, electrons are transferred from NADPH to FAD by a two-electron transfer. Reduced FAD is then reoxidized in sequential single-electron transfers to the iron center of the inner heme. Oxidase activity is lost when the FAD is removed, e.g., by solubilization in detergents, but can be restored when FAD is added back (Light et al. 1981). Since the iron of the heme can only accept one electron at a time, the inner heme must donate its electron to the outer heme before the second electron can be accepted from the now partially reduced flavin, FADH. Low-potential flavoproteins may redox cycle as one-electron carriers. In that case, the oxidized form of FAD would cycle between the semi-quinone form ($FADH^{\cdot}$) and the fully reduced form ($FADH_2$). This scenario would require dimerization of two NOXs for functional NOX activity (Mayhew et al. 1996; Vignais 2002). Transfer of the electron from the inner heme to the outer heme is actually against the measured electromotive force between these two groups; hence, to create an energetically favorable state, oxygen must be bound to the outer heme to accept the electron. The transfer of the electron from the heme group to oxygen is not well understood. EPR studies indicate that all iron coordination sites are normally occupied, a situation that would not leave room for oxygen binding (Fujii et al. 1995; Isogai et al. 1995). However, upon addition of arachidonic acid, an activator of $gp91^{phox}$, a different EPR signal indicative of a pentacoordinated configuration of the heme ion was obtained, suggesting that under conditions of stimulation the heme group may direly interact with oxygen (Doussiere et al. 1996). Conformational changes in the NOX are anticipated to be important for switching the molecule from an inactive to an active form. In its active form, the phagocyte NOX is an electron transfer chain that results from a biological assembly of cytosolic regulatory factors ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac) on the membrane cytochrome b_{558} formed by two subunits: $gp91^{phox}$ and $p22^{phox}$ (Cross and Segal 2004; Nauseef 2004). It is assumed that initiation of electron flow is the consequence of a conformational change in $gp91^{phox}$ induced by its interaction with $p67^{phox}$ (Mizrahi et al. 2006). Mechanisms that bring about conformational changes in the NOX structure are a recurrent theme in its regulation, e.g., during activation of NOX5 and Atrboh by calcium as will be discussed below.

5 NOX Inhibitors

Ongoing oxidative basal metabolism, photosynthesis and many enzymatic activities produce ROS. Thus, specific inhibitors have been sought with the ability to identify the contribution of NOX activity to the redox milieu. However, at present, no specific NOX inhibitors exist, although the most commonly used NOX inhibitor, iodonium-derivative diphenylene iodonium (DPI), has great utility if its limitations are taken

into account. Human neutrophil NOX is not inhibited by DPI pretreatment in the absence of an activating stimulus. It is likely that a reduced redox center in the oxidase could serve as electron donor to DPI to form a DPI radical. Inhibition occurs after direct phenylation of the redox cofactor or of other reactive groups in the area (Odonnell et al. 1993). Consistent with this, adducts were formed between photoreduced flavin and the inhibitor DPI. Furthermore, in pig neutrophil membranes, the quantity of recoverable intact flavin was greatly diminished when NADPH was present indicating that the flavin may be the site of DPI activation. However, it has been shown that iodonium compounds decrease the absorbance of the heme Soret peak in neutrophil membranes incubated with NADPH. The decrease was correlated with the loss of oxidase activity (Doussiere et al. 1999). Thus, the heme groups may be a secondary site of DPI action. Importantly, as suggested by the mechanism of action, DPI is a nonspecific inhibitor of many different electron transporters. It inhibits not only all the NOX isoforms, but also nitric oxide synthase (Stuehr et al. 1991), plant molybdenum-cofactor flavin-containing enzymes, xanthine dehydrogenase (XDH) (Yesbergenova et al. 2005), xanthine oxidase, mitochondrial complex I (Hutchinson et al. 2007), and cytochrome *P*-450 reductase (Zhukov and Ingelman-Sundberg 1999). The use of multiple inhibitors is thus necessary to get an indication of the relative contribution of NOXs to a particular milieu of reactive oxygen production (Yesbergenova et al. 2005).

6 Calcium-Binding Domains in RBOH Proteins

The canonical EF-hand consists of two alpha helices positioned roughly perpendicular to one another and linked by a short loop region of about 12 amino acids. The loop structure of the EF-hand binds calcium ions with residues positioned at 1, 3, 5, 7, 9, and 12 (X, Y, Z, -X, -Y, -Z). The positive calcium ion is complexed by charge interaction through negatively charged oxygen-containing side chains, such as aspartate and glutamate, that appear invariantly in the twelfth position and provide two oxygens for calcium binding (bidentate ligand). The sixth residue is invariantly a glycine that facilitates conformational bending requirements of the backbone. The remaining residues are typically hydrophobic and support a hydrophobic core that stabilizes the two helices.

All RBOH contain two EF-hand motifs where the EF-hand located closest to the N-terminal motif tends to be canonical. AtrbohI is exceptional, lacking canonical EF-hands in both structures (Fig. 4). The second EF-hand motif in all Atrboh is non-canonical lacking the invariant aspartate or glutamate at position 12 (-Z) as illustrated in Fig 4 (Kawahara et al. 2007). AtrbohF was shown to bind calcium (Keller et al. 1998) and calcium was shown to activate partially purified plant membrane fractions (Sagi and Fluhr 2001). Further insight into the binding properties of RBOH was obtained by examining a recombinant polypeptide of 83 amino acid size of the N-terminal region of AtrbohD. Calcium-dependent conformational changes were followed by fluorescence spectroscopy taking advantage of the presence of

	EF-1		EF-II[1]
	X Y Z-Y-X –Z		X Y Z-Y-X -Z
AtrbohA	[234]DKDSDGRLNEAE	32	DPYHYGYIMIEN
AtrbohB	[184]DKNLDGRITGDE	32	DRDNLGYIELHN
AtrbohC	[239]DKDADGRLTEDE	32	DPDNIGYIMLES
AtrbohD	[266]DKDEDGRVTEEE	32	DPDNAGFIMIEN
AtrbohE	[281]DSNEDGKITREE	32	DPENFGYIELWQ
AtrbohF	[277]DKNEDGRITEEE	32	DPERLGYIELWQ
AtrbohG	[189]DKDSDGRLTEDE	32	DPDHMGYIMMES
AtrbohH	[208]DKNGDGKLTEEE	32	DPDHKGYIEMWQ
AtrbohI	[265]CYQLSSNLVKHI	32	APDGLYSQYIEL
AtrbohJ	[218]DKDGDGKLTEEE	32	DPNEQGYIEMWQ
	*		* ^

Fig. 4 Atrboh EF-hand motif alignment in Atrboh genes. The sequences are deduced by comparison to canonical EF-hand domains and the NOX5 sequence. The positions of critical calcium binding amino acids are indicated by *X,Y,Z-Y-X* and *-Z*, and the amino acid position and spacing between domains are indicated. The invariant glycine at position 6 is indicated by ∗ and the non-canonical -Z residue in the second EF-hand is indicated by ^

resident tyrosine residues as indicators of such changes (Ogasawara et al. 2008). The results showed that a level of 0.1 mM of calcium induced 50% of the observable conformational change. Considering that cellular calcium levels are orders of magnitude lower, the EF hands in this context (i.e., a short polypeptide) indicate only moderate sensitivity to calcium. When the non-canonical asparagine in the second EF-hand at the twelfth position was mutated to the acidic amino acid (N321E), a higher affinity to calcium was obtained indicating a difference in the affinity of each domain for calcium. However, when the EF-hands are examined in the context of the complete polypeptide, i.e., in a human cell line transfected with the full AtrbohD, a higher sensitivity to calcium was obtained. Indeed, in those cell lines, an interesting synergistic activation in the presence of the phosphatase inhibitor, calyculin A, and the calcium ionophore, ionomycin, was observed. Calyculin A leads to a higher phosphorylation state of the polypeptide. As the level of cellular calcium under these conditions was measured at 10^{-7} M this indicates a higher affinity to calcium due to either the phosphorylation of the protein, or that EF hands in the context of the full protein bind calcium more efficiently.

The NOX5 prototype could provide an important paradigm for understanding plant RBOH biology. NOX5 contains three canonical EF-hands whilst the fourth at the N-terminus is non-canonical. In a cell-free system, its activity is entirely dependent on calcium (Banfi et al. 2004b). Flow dialysis experiments established binding for all four sites with a tenfold difference in affinity assigned to the non-canonical structure. Interestingly, when tyrosine fluorescence was used to measure conformational change, such change occurred when both the N and C terminal fragments were together, indicating the necessity of their interaction. The researchers took advantage of the fact that a single cysteine was present in the alpha helical

region flanking EF-III and noted that its titratable reduction by DTNB was accelerated in the presence of calcium, suggesting a more accessible conformation in that state. Indeed, when TNS, a hydrophobic probe, was used to measure the degree of hydrophobic exposure in the polypeptide, a large increase in its interaction was observed after calcium addition. Changes in hydrophobicity were further established by showing calcium-dependent binding of the protein melittin (Banfi et al. 2004b). Melittin, a 26 amino acid peptide, makes up 50% of the dry weight of bee venom; its hydrophobicity acts to destroy the victims blood cells by breaking up their membranes. Melittin was shown to form a complex with the NOX5 N-terminal fragment only in the presence of calcium. The calcium-dependent formation of a hydrophobic core is reminiscent of calcium binding to calmodulin that results in exposure of a deep hydrophobic cavity essential for recognition of calmodulin target protein. Interestingly, the binding of melittin was shown to inhibit NOX5 ROS production implying that the N-terminal region could interact with the 'business end' of NOX5. Indeed, recombinant N-terminal fragment could be shown to bind in a calcium-dependent manner to recombinant C-terminal containing the FAD and NADPH domains (Banfi et al. 2004b). Thus, the amino terminus of NOX5 operates in a manner analogous to calmodulin by promoting interaction with the C-terminal domain which then facilitates electron delivery and ROS production. The C-terminus of NOX5 was shown to contain a canonical calmodulin binding site of the so-called 1-14 CaMBD subclass that contains bulky hydrophobic residues in positions 1, 5, and 8 with a basic residue in position 13 (Fig. 5). This domain interacts with the N-terminal region and appears to increase the sensitivity of NOX5 enzymatic activity to calcium (Tirone and Cox 2007). Inspection of the RBOH plant sequences reveals that only AtrbohI sequence fills these criteria. Interestingly, only AtrbohI completely lacks canonical EF-hands sequence in its N-terminus, which may indicate alternative modes of regulation for this protein.

```
.........1...5..8...13.....NADPH
NOX5-    92WSKVFQKVAAEKKGK-VQVFFCG
AtrbohA- WRSVFKRIAVNHPKTRVGVFYCG
AtrbohB- WRSVFKHVAVNHVNQRVGVFYCG
AtrbohC- WRNVYKRIAMDHPNTKVGVFYCG
AtrbohD- WRQVYKKIAVQHPGKRIGVFYCG
AtrbohE- WKEVFSSIARKHPNSTVGVFYCG
AtrbohF- WKKVLTKLSSKHCNARIGVFYCG
AtrbohG- WKNVYKQIAMDHPGANVGVFYCG
AtrbohI- WKKVLSKISTKHRNARIGVFYCG
AtrbohJ- WRKVFSELSNKHETSRIGVFYCG
```

Fig. 5 Putative calmodulin binding motifs in human NOX5 and Atrboh genes. Evidence for calmodulin binding in NOX5 is presented in the text and putative homologous regions are indicated for Atrboh genes. The sequence of AtrboH lacks homology to the other Atrboh in this region and is not included

7 Phosphorylation of RBOH Potentiates Calcium Responses

The relatively high levels of calcium required to activate NOX5 raised the question of its physiological relevance. To address this, COS-7 cells that do not normally experience elevated calcium levels, were transformed by NOX5 and examined for activity (Jagnandan et al. 2007). In this case, the addition of 12-myristate 13-acetate (PMA) was necessary to activate ROS production. The activity was greatly potentiated by the addition of ionomycin in a calcium-dependent manner. PMA was shown to induce phosphorylation of NOX5. A cluster of potential PKC substrate residues was identified at S486, S490, T494, and S498 all localized to the FAD domain region. Site-specific mutagenesis to alanine was carried out, and PMA activation and potentiation of the response to calcium was examined. While S486A showed no effect, mutagenesis of the other potential sites showed additive inhibition of the response. Indeed, mutations of T494E/S498E produced a phosphomimetic response in the absence of PMA. Interestingly, at the equivalent NOX5 T494 position, a conserved serine is present in AtrbohB, D, and F.

Arabidopsis leaves with functional FLS2 receptor rapidly produce ROS in response to the application of flagellin elicitor, flg22 (Gomez-Gomez et al. 1999). In a proteomic phosphorylation study, amino acid sites were recovered that show differential phosphorylation after xylanase or flg22 application these include; ^{35}GAFpSGPLGRPK, ^{341}ILpSQMLSQK, and ^{161}TSpSAAIHALK and indicate potential RBOH regulatory sites (Benschop et al. 2007). Inspection of the data in supplementary Table III (Benschop et al. 2007) reveals that at least in the case of xylanase treatment, an additional peptide ^{697}PPpTAGKpS showed differential phosphorylation. This peptide serine is at a conserved position similar to NOX5 T494. Whether it serves the same function in Atrboh is unknown. The sites in AtrbohD, S343 and S347, were confirmed to undergo phosphorylation after treatment with flg22 elicitor (Nuhse et al. 2007). These sites are conserved in several other Atrboh members and are indicated in Fig 2. Importantly, when they were mutated to alanine and transformed into the AtrbohD mutant, the leaf strips no longer responded to flg22 (Nuhse et al. 2007). None of these sites overlap with the phosphorylation site found for NOX5. Thus, AtrbohD has been shown to undergo elicitor-augmented phosphorylation at conserved and unique sites that contribute to its activity.

Kinases that may be directly responsible for RBOH regulation have been identified in potato. Using an anti-phosphopeptide antibody, Ser-82 and Ser-97, that are present in the N terminus of potato StrbohB, were identified as potential phosphorylation sites and Ser-82 was phosphorylated by pathogen signals in planta (Kobayashi et al. 2007). Two calcium-dependent protein kinases, StCDPK4 and StCDPK5, were shown to specifically phosphorylate these serines in a calcium-dependent manner. Significantly, in *N. benthamiana* the over-expression of the constitutively active mutant of StCDPK5 phosphorylated Ser-82 of StrbohB and induced ROS accumulation that depended on the presence of the homologous gene NbrbohB. In leaves where NbrbohB expression was abrogated, the loss of function could be complemented by expression of wild-type potato StrbohB but not by a mutant (S82A/S97A). These results suggest that calcium-dependent protein kinases are

conserved and responsible for phosphorylation of the StrbohB N-terminal region and that this activity can regulate the oxidative burst. The positions of these residues fall on conserved serines present in Atrboh (S127 and S148) that have not been shown to be phosphorylated after elicitor treatment (above). The lack of identity in phosphorylated residues may be a result of comparison of non-orthologous genes or indicate inter-specific differences. However, in both *Arabidopsis* and potato, it seems evident that phosphorylation of the N-terminus serves to potentiate RBOH activity.

8 Small G Proteins Interact with RBOH

Full activity of human Nox1, -2, and -3 requires the direct action of a Rac GTPase. A Rac interaction site was discovered in human NOX2 juxtaposed to the first NADPH interacting site within amino acids 419–430 of NOX2 (Fig. 3) (Kao et al. 2008). Human NOX5 does not show a requirement for Rac and all the amino acids that are critical for Rac interaction in NOX2 are absent. It is therefore unlikely that this region is used for this purpose in plant RBOH. Rac/Rop small GTPases are prevalent in the plant kingdom and are part of multigene families (11 in *Arabidopsps*; 9 in rice) (Gu et al. 2004; Wong et al. 2007). OsRac1, which is located in the plasma membrane, is involved in Osrboh-dependent ROS production and cell death initiation during defense signaling (Kawasaki et al. 1999; Ono et al. 2001). Indeed, constitutively active and dominant negative forms of the small GTP-binding protein were shown to directly modulate ROS production. Furthermore, ROS scavengers such as metallothionein were reciprocally downregulated by Rac showing that OsRac1 plays a critical role in regulating the cellular redox milieu (Wong et al. 2004). The constitutively active (CA) form of OsRac2 and OsRac7, but not the dominant negative (DN) form, interacted with four different rice and two potato RBOH N-terminal fragments (Wong et al. 2007). Furthermore, transient agroinfiltration of *N. benthamiana* leaves produced ROS when the CA form was used. More evidence for the involvement of small GTPases in RBOH upregulation was provided by the phosphatidic acid-dependent induction of ROS in *Arabidopsis* cells (Park et al. 2004). In contrast to the results that Rac plays a positive role in RBOH induction, tobacco cells transformed with sense constructs of Ntrac5 or the constitutive active GTP form, Ntrac5V15, showed a decrease in the production of ROS after elicitation with cryptogein (Morel et al. 2004). Hence, Ntrac5 could be considered as a negative regulator of NtrbohD. Taken together, there is compelling evidence that Rac modulates RBOH and we may expect to find that different Rac types either induce or inhibit RBOH activity.

In animal NOX 1, 2, and 4, Rac binding was localized to the NADPH interacting region. In contrast, in rice, both N-terminal EF-hands were essential for Rac interaction and the full two hybrid interaction was obtained only when a considerable length of N-terminal region was used. In vitro examination showed that OsrbohB (residues 138–313) binds the GTP-binding form of OsRac1 (in the presence GMPPCP

a nonhydrolyzable Rac substrate), apparently the binding of Rac to Osrboh is very sensitive to the character of its bound cofactor. In a fluorescent resonance energy transfer (FRET) system in which both interacting partners were fused to different fluochromes on the same polyprotein, a strong FRET signal was obtained with the CA but not DN OsRAC1 form. When calcium was added, this FRET signal decreased suggesting a negative interaction between calcium and OsRac binding (Wong et al. 2007). The authors therefore suggested a scenario in which calcium, at low levels, indirectly activates RBOH through CDPK phosphorylation, subsequently producing a conformational status that induces Rac binding and RBOH activation. Finally, continued cellular calcium buildup would disrupt Rac binding and return RBOH binding to a quiescent state. This scenario is seemingly in conflict with other evidence presented above, including direct activation of human NOX5 by calcium (Banfi et al. 2004b), the fact that calcium serves as a direct positive activator for partially purified tobacco membranes (Sagi and Fluhr 2001), and that in a heterologous expression system recombinant AtrbohD is activated by calcium (Ogasawara et al. 2008). It is thus likely that both calcium and Rac are positive – although mutually exclusive – activators permitting multiple inputs to impact on RBOH activity.

9 Membrane Microdomain Milieu of RBOH

Cellular fractionation of plant tissue indicates that RBOH proteins are found in plasmalemma membrane (Sagi and Fluhr 2001; Simon-Plas et al. 2002). However, the plasmalemma is not a uniform entity. Thus, NtrbohD was found to be enriched in tobacco BY2 cells on chemically distinct membrane microdomains called lipid rafts (Mongrand et al. 2004). Such rafts form a distinct lipid phase within the membrane and can be isolated due to their insolubility in nonionic detergents, e.g. Triton X-100. Plant lipid rafts are similar to animal lipid rafts and include a four- to fivefold enrichment of sterols and sphingolipids as their major components but are depleted for phospholipids (Borner et al. 2005; Grennan 2007). An exhaustive survey of tobacco BY2 cells identified 145 proteins in the detergent resistant membrane (DRM) fraction (Morel et al. 2006). This work confirmed that NtrbohD was present in lipid rafts as well as its possible regulator, the small G protein Rac5. Other proteins associated with hypersensitive response and pathogen resistance, such as HIN9, HIN18, and members of the gene family HIR, were also detected (Nadimpalli et al. 2000; Takahashi et al. 2004). In contrast, in an analysis of the *Medicago* root plasma membrane, RBOH was not detected, instead a slew of redox-generating proteins were recovered which could fill the role of ROS generation in root tissue (Lefebvre et al. 2007). Lipid rafts seem to be the focus of particular cellular events, i.e., while transport proteins are under-represented, others, like proteins that coordinate signaling, are enriched, including cellular trafficking, cell wall metabolism, and RBOH.

The localization of RBOH into lipid rafts can have far-meaning ramifications in RBOH biology. This can be discerned from following the dynamic changes in

positioning of human NOX in membranes during its activation. In general, the NOX proteins are resistant to solubilization in Triton X-100. Following phagocyte activation, cholesterol-enriched microdomains act to recruit and/or to organize the cytosolic NOX factors in the assembly of the active NOX (Vilhardt and van Deurs 2004). Kinetic analysis of the activation of NOX in neutrophils implies that the onset, but not the maximal rate, of enzyme activity is determined by its presence on lipid rafts (Shao et al. 2003). However, different NOXs or cell types may alter the microdomain localization. Thus, NOX4 is highly expressed in cardiovascular tissue and ROS is implicated in cardiovascular diseases, including hypertension (Ellmark et al. 2005). The D1-like receptor agonist, fenoldopam, used for hypertension treatment dispersed NOX subunits of human renal proximal tubule (hRPT) cells that are found within lipid rafts and resulted in the reduction of ROS production, suggesting a novel mode of action for this drug. In contrast, cholesterol depletion caused the translocation of NOX subunits out of the lipid rafts and increased measurable ROS. Thus, in human renal proximal tubule cells, lipid rafts maintain NOX in a quiescent state suggesting that NOX micro-localization is important for its regulation (Han et al. 2008). In contrast, in endothelial cells, the activation by TNF-α stimulates lipid raft clustering of NOX2 components. This is important, as NOX2 positioning facilitates endothelial cell migration in the process of tissue healing (Ikeda et al. 2005; Ushio-Fukai 2006). In this context, caveolae invaginations of the plasma membrane of endothelial cells recruit NOX1 under unstimulated conditions and undergo activation by further recruitment of Rac1 after stimulation. The dynamic properties of lipid rafts that contribute to NOX activation have important ramifications for understanding RBOH biology.

10 Extracellular and Intracellular Localization of NOX and RBOH

Spatial, temporal, and quantitative components of ROS appearance dictate its biological significance during signaling. Therefore, the localization of NOX activity is crucial for its function. Cellular fractionation of plant tissue indicates that RBOH proteins are found in the plasmalemma membrane (Sagi and Fluhr 2001; Simon-Plas et al. 2002). Furthermore, recombinant GFP polypeptides of potato StrbohA and StrbohB localized to onion peel plasmalemma membranes during transient transformation (Kobayashi et al. 2006). The distribution on the membrane can be asymmetric. For example, RBOH activity in AtrbohC-dependent ROS signaling in root hair growth is distributed mainly to the tip (Carol et al. 2005; Foreman et al. 2003), and in RBOH involvement in xylem differentiation to one particular cell side (Barcelo 2005).

When situated on the plasmalemma, RBOH enzymatic activity would mediate the vectoral transfer of electrons to produce short-lived superoxides extracellularly. The locally produced signals do not propagate more then a few cell lengths from its source (Allan and Fluhr 1997). While extracellular functions for such ROS are

evident (e.g. cell wall cross-linking) it is likely that the ROS signals generated by RBOH are processed intracellularly (Sagi et al. 2004; Sagi and Fluhr 2006). The relationship between externally produced superoxide product to modulation of the intracellular ROS signal are fundamental questions that remain to be answered. Given the importance of membrane topology in ROS production and subsequent redox signaling a thorough understanding of where O_2^- is converted to H_2O_2 is necessary. For example, the superoxide product is membrane impermeable in animals due to its negative charge in ambient conditions of pH (*p*Ka of superoxide is 4.8, e.g. blood pH is 7.4). However, under conditions of exceptionally low pH, the superoxide can be protonated and as such has been shown to be capable of crossing a yeast membrane (Wallace et al. 2004). In plants, the physiological range of extracellular pH is 5; in this case 16% of the superoxide would be in the membrane permeable hydroperoxyl (HO_2•) form. Thus, the external pH status could moderate the compartmentalization of superoxides produced by RBOH outside the membrane. Once having entered, the cytoplasmic SOD would convert the incoming superoxide to hydrogen peroxide. This scenario does not seem to provide efficient use of the NOX product. Alternatively, the superoxide could be converted extracellularly by an unknown SOD and the resultant product, H_2O_2, would then be freely permeable (Fig. 6). Evidence in support of the presence of extracellular SOD was found in secreted tobacco nectar that contains large amounts of hydrogen peroxide. The hydrogen peroxide is supplied in-part by the tandem action of RBOH and an extracellular germin-like superoxide dismutase protein called Nectarin I (Carter and Thornburg 2004). Other extracellular SOD activities are known, including a high isoelectric point-SOD isoform (hipI-SOD) that has been identified in the secondary cell wall of *Zinnia elegans* and pine (Karlsson et al. 2005; Karpinska et al. 2001).

Evidence from human NOX biology shows that they are not situated solely in the outer membrane; indeed, NADPH subunits have been identified in diverse intracellular locations. Within the cell, NOX5, the form most structurally related to RBOH, was detected in detergent-resistant microdomains of the endoplasmic reticulum. Generally, a strong perinuclear staining, including an extensive network of branching tubules that are reminiscent of localization to the endoplasmic reticulum as well as the plasma membrane, was observed. In the case of NOX5 over-expressing cells, it is difficult to distinguish between ER retention due to NOX5 over-expression and a true ER localization. (Jagnandan et al. 2007; Serrander et al. 2007).

The phagocyte-NOX2 expressed in endothelial cells differs from the neutrophil enzyme in that it exhibits activity even in the absence of agonist stimulation. When the subcellular location of oxidase subunits and activity were followed, 50% of the total activity and protein were found by fractionation to be in a ‘low g’ nuclear fraction (Li and Shah 2002). These results indicate that, in contrast to the neutrophil enzyme, a substantial proportion of the NOX of unstimulated endothelial cells exists as a preassembled intracellular complex associated with the cytoskeleton. The use of GFP-labeled recombinant proteins has provided additional visible support for internal, i.e., endoplasmic reticulum, localization for active NOXs particularly in the nuclear and ER regions (Ambasta et al. 2004; Li and Shah 2002; Martyn et al. 2006; Van Buul et al. 2005).

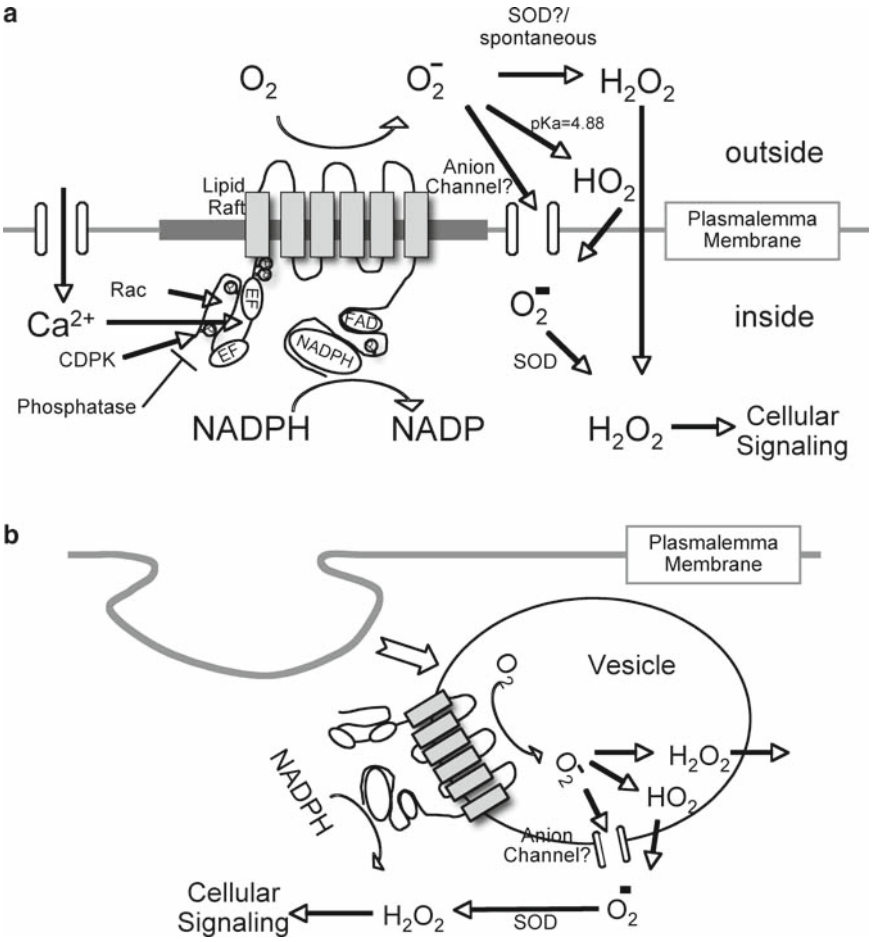

Fig. 6 Mode of RBOH activity. RBOH is shown situated on the plasmallema (**a**) or on a vesicle (**b**). In each case, the superoxide signal must diffuse into the intracellular space to induce redox-dependent signaling. RBOH interacts with calcium ions by binding through the EF hands as well as with Rac by binding within the N-terminal region. CDPK-like kinases phosphorylate the N-terminal region or together with other kinases at other regions, leading to potentiation of RBOH activity. The superoxide product will dismutate spontaneously to H_2O_2 or diffuse through the membrane via an anionic channel or diffuse through the membrane in a protonated form. Within the cell, the ROS product facilitates cellular signaling

Endocytosis is associated with downregulation of receptor-mediated signaling at the plasma membrane. However, it can also play an important role in the specificity and amplification of membrane-initiated receptor signals (Sorkin and von Zastrow 2002). NOXs are known to be internalized during activation by endocytosis. For example, H_2O_2-mediated redox change is a key mediator in IL-1β and TNF-α activation of NF-κB. The source of H_2O_2 was found to be provided by NOX2 that is situated in activated endosomes formed after stimulation by IL-1β (Li et al. 2006).

In this scenario, the local increases in H_2O_2 facilitates the redox-dependent association of TRAF6 with the receptor complex on ligand-activated endosomes, leading to activation of downstream IKK kinases and ultimately of NF-κB. Seemingly, upon membrane invagination, topologically the NOXs would produce superoxides that tend to be trapped in such endosomes. However, in mammary epithelial tumor cells that produce superoxides in their endosomes, an anion channel was found to play a role in endosome superoxide transport, thus providing an avenue for superoxide escape. In addition, activated endosomes recruited human SOD1 suggesting the rapid generation of H_2O_2 from exiting dismutating O_2^- at the endosomal surface (Mumbengegwi et al. 2008). It remains to be seen if such scenarios for ROS membrane translocation and efficient superoxide processing are prevalent elsewhere.

Only indirect evidence exists in plants for internalization of RBOH. Cryptogein, a proteinaceous elicitor, produced by the pathogenic fungus *Phytophthora cryptogea*, induces a hypersensitive response in tobacco (Binet et al. 2001; Ricci et al. 1989). An unidentified plasma membrane receptor leads to a cascade of events, including calcium influx, potassium and chloride efflux, cytosolic acidification, and plasma membrane depolarization that lead to RBOH activation (Bourque et al. 1999; Pugin et al. 1997; Simon-Plas et al. 2002). The production of apoplastic ROS in the oxidative defense-related burst promoted by cryptogein and other pathogens is biphasic: a rapid and transient production of ROS, which occurs within minutes of the perception of pathogens, and a second much later phase of ROS production within hours, which is specific to hypersensitive response-inducing pathogens (Grant and Loake 2000). The use of Amplex Red reagents, that can report real-time H_2O_2 accumulation in tobacco BY-2 cell populations, showed an internal signal for H_2O_2 that developed more rapidly than the external apoplastic signal. Thus, the ROS signal was actually initiated within the cells (Ashtamker et al. 2007). Furthermore, the fluorescent probe for H_2O_2, 2′,7′-dichlorofluorescin diacetate (DCF), showed signal development first in the nuclear region and only after a short delay in the cell periphery. Those results are consistent with ROS originating at intracellular sites. Indeed, subcellular accumulation of ROS was evident in the cytoplasm, endoplasmic reticulum (ER), and the nuclear region, implying diverse localization for tobacco RBOH (Ashtamker et al. 2007). These results are consistent with the observation that cryptogein-stimulated clathrin-mediated endocytosis in BY-2 cells was correlated with ROS production (Leborgne-Castel et al. 2008). Similarly, in *Arabidopsis* roots, RBOH-dependent hydrogen peroxide is produced during salt stress. During salt stress, ROS-loaded vesicles were noted to undergo trafficking to the central vacuole (Leshem et al. 2006).

11 Conclusion

NOXs are tethered to perform multiple signaling tasks. Some of what we have learned about the exquisite temporal and spatial control is depicted in Fig. 6. RBOH is localized to dynamic lipid rafts in the plasmalemma membrane and likely also