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Respiratory burst oxidases: the engines of ROS signaling

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Reactive oxygen species (ROS) play a key signal transduction role in cells. They are involved in the regulation of growth, development, responses to environmental stimuli and cell death. The level of ROS in cells is determined by interplay between ROS producing pathways and ROS scavenging mechanisms, part of the ROS gene network of plants. Recent studies identified respiratory burst oxidase homologues (RBOHs) as key signaling nodes in the ROS gene network of plants integrating a multitude of signal transduction pathways with ROS signaling. The ability of RBOHs to integrate calcium signaling and protein phosphorylation with ROS production, coupled with genetic studies demonstrating their involvement in many different biological processes in cells, places RBOHs at the center of the ROS network of cells and demonstrate their important function in plants.

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Introduction

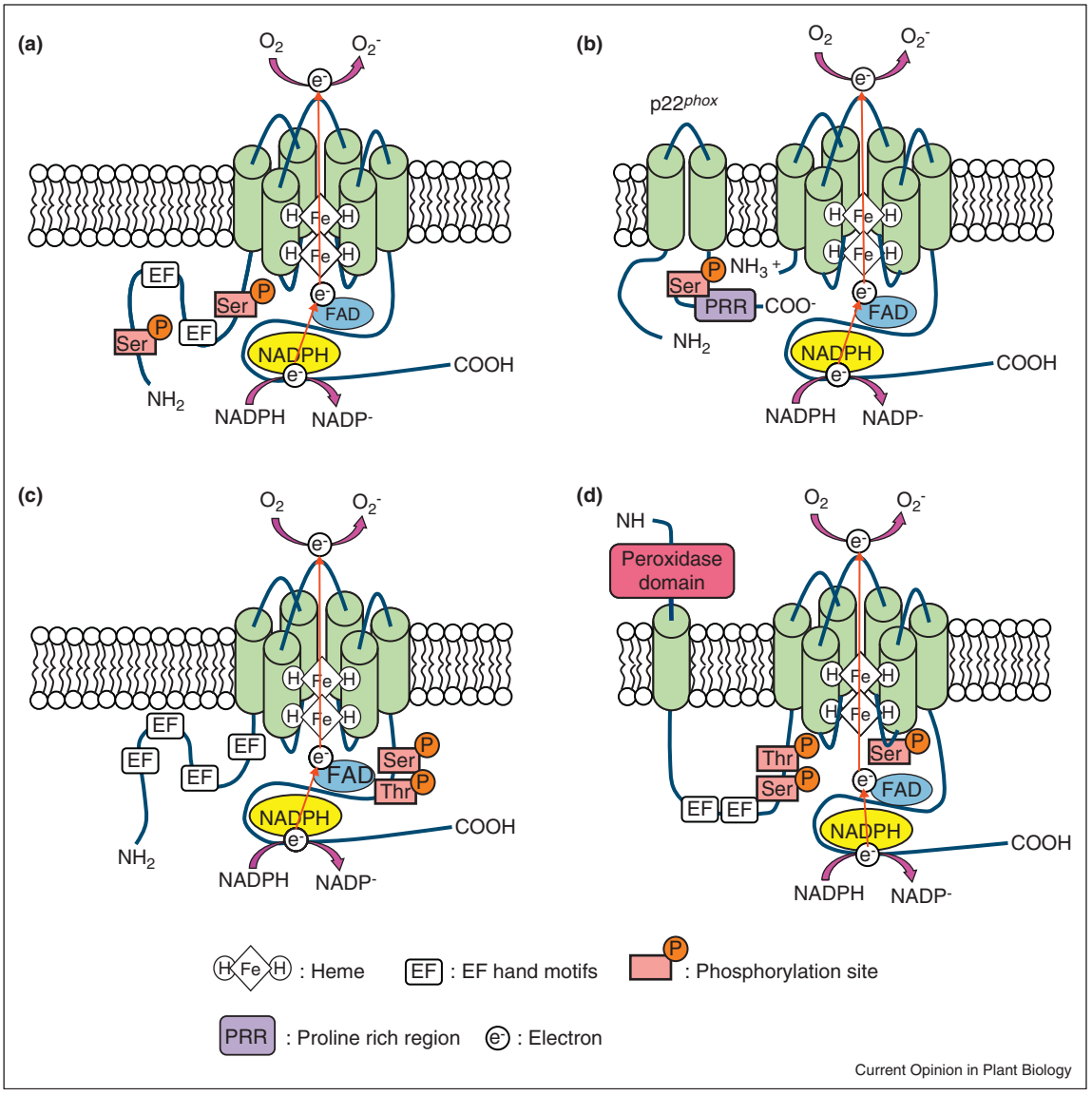
The reactive oxygen species (ROS) signaling network is an evolutionary conserved signal transduction network found in all aerobic organisms [1]. It uses ROS such as singlet oxygen ($^1O_2$), superoxide (O_2^-) and/or hydrogen peroxide (H_2O_2) as signal transduction molecules to control a large array of biological processes ranging from the regulation of development and growth to responses to biotic and/or abiotic stimuli [1]. Although early research into ROS metabolism has focused on their toxic potential [2], most recent studies have focused on ROS as important signal transduction molecules. The key to using ROS as signaling molecules appears to be the capacity of cells to detoxify or scavenge them using a network of ROS scavenging enzymes found in almost all cellular compartments [3,4]. This network enables cells to maintain a non-toxic steady-state level of ROS, while allowing for the transient accumulation of ROS in particular subcellular locations, that act as signals [4]. Countering this network of ROS detoxifying enzymes are different cellular pathways that produce ROS. These include basic cellular process such as photosynthesis, respiration and photorespiration, that produce ROS as an unavoidable by-product, and a network of ROS producing enzymes that include glucose oxidase, xanthine oxidase and different classical plant peroxidases [3]. A key player in this network of ROS producing enzymes is the specialized respiratory burst or NADPH oxidase enzyme [5]. In this review we summarize recent research into the role of respiratory burst oxidase homologue (RBOH) proteins in plants.

Plant RBOH-NADPH oxidase function and regulation

The plant *Rboh* constitutes a multigenic family with ten *AtRboh* genes in the model plant Arabidopsis [4,5]. All RBOH proteins present the same domain structure, with a core C-terminal region that contains the transmembrane domains and the functional oxidase domain responsible for superoxide generation (Figure 1). However, plant RBOHs have an additional N-terminal region, absent in the phagocyte oxidase gp91^{phox}, but present in other animal NADPH oxidases (NOXs) such as NOX5 and Duox ([5,6•]; Figure 1). This N-terminal extension contains regulatory regions such as calcium-binding EF-hands and phosphorylation domains important for the function of the plant oxidases.

In recent years, several genetic studies revealed that plant *Rbohs* play a multitude of different signaling functions (Figure 2). *Rboh*-dependent ROS has been associated with the establishment of plant defences in response to pathogens, often in association with the hypersensitive response [7–9]. Plant NADPH oxidases also mediate other plant biotic interactions such as the establishment of functional symbiotic nodules in *Medicago* [10•]. Moreover, *Rbohs* regulate signaling in response to abiotic stresses such as heat, drought, cold, high-light (HL) intensity, salinity or wounding [11,12••]. In addition, plant NADPH oxidase regulates developmental programs such as polarized cell expansion in root hair formation and pollen tip growth

Figure 1

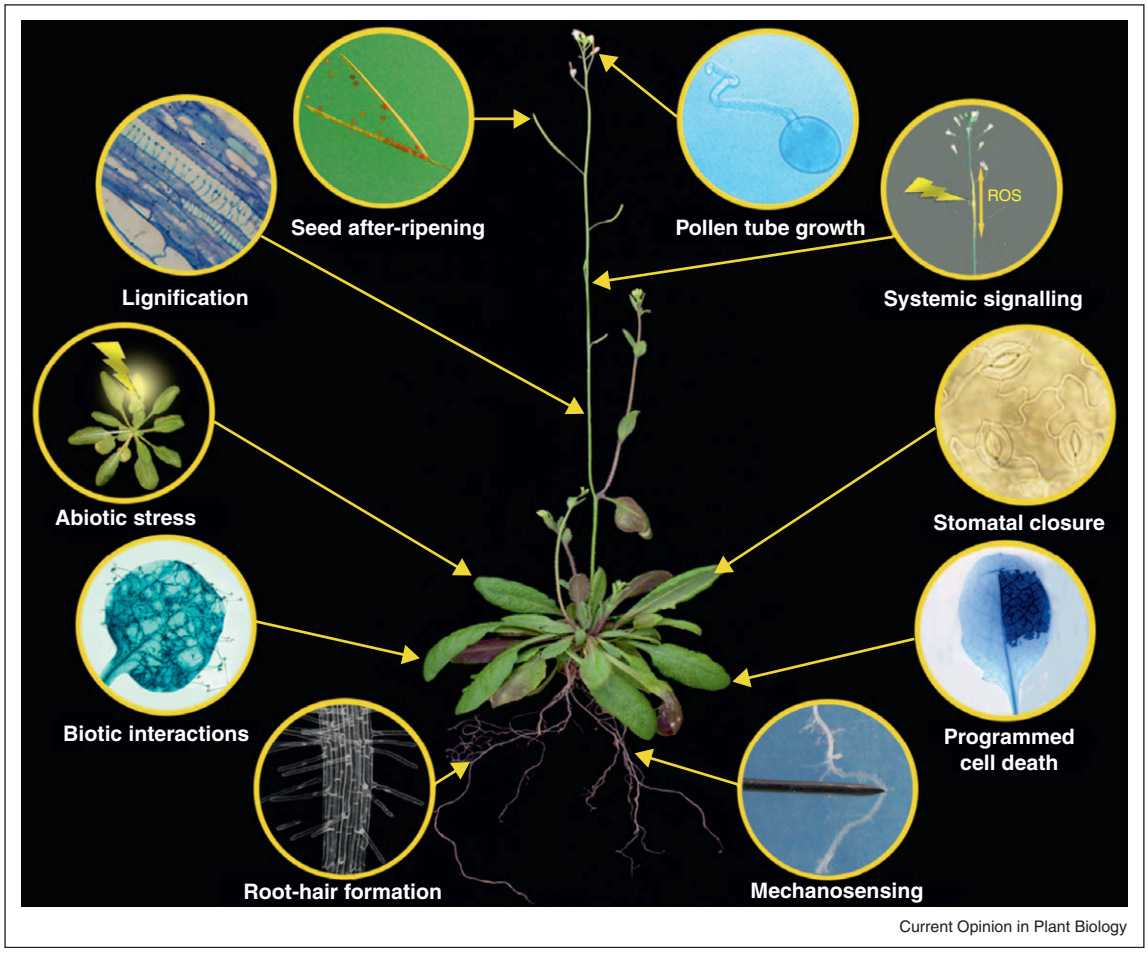


Structure of NADPH oxidases in plants and mammals.
A conserved C-terminal core region consisting of 6 transmembrane α-helices (cylinders) and 2 heme groups (indicated by 'H' and 'Fe') is found in all RBOHs. **(a)** Plant RBOH; Two EF hand motifs are present in the N-terminal region. **(b)** Mammalian Nox1–Nox4; This Nox subfamily forms heterodimer with $p22^{phox}$ that contains 2 transmembrane α-helices and a proline rich region (PRR). **(c)** Mammalian Nox5; Four EF hand motifs are present in the N-terminal region. **(d)** Mammalian Duox; Peroxidase domain in the N-terminal region is involved in H_2O_2 generation. Phosphorylation sites were identified by [28,58–60].

[13,14], or seed ripening [15•]. Often, the same *Rboh* mediates multiple functions. *AtRbohC* regulates root hair formation [13] and mechanosensing [16••]. Arabidopsis *AtRbohD*, the most highly expressed Arabidopsis NADPH oxidase, mediates many processes such as pathogen responses, stomatal closure, systemic signaling in response to abiotic stresses or lignification [7,11,12••,17•,18••]. Some responses are mediated by a single specific *Rboh*, such as systemic signaling or lignification that are mediated by *AtRbohD* [12••,18••] or seed ripening by *AtRbohB* [15•]. On the contrary, there is evidence for functional redundancy, since, for example, the double mutant *atrbohD/atrbohF* shows enhanced phenotypes compared to the individual mutants in pathogen response, cell death regulation, or stomatal closure [7,11,19•]. This multiplicity of actions implies a precise regulation of spatial and temporal *Rboh* function.

Immunolocalization studies indicated that RBOH proteins localize at the plasma membrane [20,21]. However, the

Figure 2



Multiplicity of functions of the plant *Rboh*-NADPH oxidases.
ROS produced by RBOH proteins mediate multiple processes in plants: seed after-ripening [15•]; lignification [17•]; abiotic stress [12••,43]; biotic interactions [7]; root hair formation [13]; mechanosensing and wound responses [12••,16••]; programmed cell death [19•]; stomatal closure [11,30••]; systemic signaling [12••]; pollen tube growth [14].

presence of RBOH proteins in particular lipid microdomains could enhance their functional specificity [1]. RBOH clusters to the apical membrane of elongating root hairs and the growing tip of pollen tubes, which could restrict ROS production during cell elongation to the growth tips [21,22•]. In addition, ROS, presumably *Rboh*-dependent, have been detected in vesicles in response to salt stress or during abscisic acid (ABA)-induced stomatal closure [23,24]. This could allow ROS trafficking between different compartments. Also, vesicle turnover was proven important for maintaining AtRBOHC at the plasma membrane during root hair formation [21]. Thus, specific subcellular localization could define the precise location of ROS accumulation and function and contribute to the specificity in function of plant NADPH oxidases.

Progress in understanding the regulation of plant NADPH oxidases has focused in recent years on how the N-terminal region of RBOH plays a crucial role in the regulation of oxidase activity [6•]. A tight relationship between *Rboh*-dependent ROS and calcium homeostasis has been revealed. A positive feedback amplification of these signals exists, with calcium binding to the RBOH EF-hands and promoting ROS production, which subsequently activate calcium channels [21,25]. Self-amplification loops involving RBOH and phosphorylation also contribute to the amplification of signals in defence and wound responses and stomatal closure [8,26,27••]. Phosphorylation of residues located at the N-terminal region of RBOH, by calcium-dependent protein kinases, implies a crosstalk between calcium and phosphorylation in the regulation of ROS production [28]. Also, calmodulin-dependent activation of mitogen-activated protein kinase 8 was shown to be essential for maintaining ROS homeostasis [29••]. In addition, RBOH can be activated by phospholipase Dα1-generated phosphatidic acid [30••], or by binding of small Rac GTPases to its N-terminal extension

[31]. Thus, there is a large repertoire of mechanisms that can contribute to the tight control of ROS production by plant *Rbohs* to allow their signaling function.

The involvement of RBOH proteins in systemic signaling

The role of NADPH-oxidases, such as the plant *Rboh* or the animal NOX, in cellular signaling and different regulatory events was recognized at the beginning of the previous decade [32]. Growing lines of evidence from mammalian systems, zebrafish and Arabidopsis suggest the involvement of NADPH oxidase-generated oxidative burst in extracellular signaling and cell-to-cell communication [12••,33•,34•]. This includes involvement of NOX2 in chemotaxis, generating ROS to ensure proper guidance and recruitment of immune cells to the site of infection in humans and mice [33•], generation of an extracellular tissue-scale H_2O_2 gradient by dual-oxidase (Doux) to attract leukocytes to a wound site in zebrafish [34•], and rapid activation of gene expression in systemic tissues in response to divergent stress stimuli mediated by *RbohD* in Arabidopsis [12••]. The ROS intercellular signal could be delivered from cell-to-cell through diffusion [34•], by exosomes, or other nano-vesicles containing high levels of H_2O_2 [35], or via oxidative burst proliferation at the apoplast [1].

Plant RBOH proteins are homologues of the $gp91^{phox}$ protein from mammalian phagocytes, which can induce oxidative burst in the absence of cytosolic components ([20,32,36]; Figure 1). Compared with their NOX homologues that function in mobile cells of the animal immune system, RBOH proteins function in plants in cells that are immobile. Despite their immobility, RBOHs were recently shown to mediate long distance signaling via a dynamic autopropagating wave of ROS that can travel at a rate of up to 8.4 cm min^{-1} [1,12••]. This ROS wave can be produced in a particular part of the plant and travel through the apoplast to the entire plant causing rapid activation of gene expression in systemic tissues [12••]. The function of *Rbohs* in mediating such long distance signals appears to be highly important for the ability of plants to rapidly induce systemic acquired acclimation (SAA) ([12••,37]; Suzuki *et al.*, unpublished). Reduced ability to accumulate local and/or systemic ROS in Arabidopsis and tomato for example compromised the systemic expression of wound-induced transcripts and activation of the heat stress response in systemic leaves [12••,37]. The autopropagation quality of ROS signaling has originally been demonstrated in 1998 by Park *et al.* in potato tubers, in which application of a fungal elicitor induced an oxidative burst in distal non-treated parts of the tissue [38]. Extracellular autoactivation of *RbohD*-induced oxidative burst was also demonstrated in Arabidopsis leaves injected with xanthine/xanthine-oxidase generating superoxide ions [19•]. Extracellular ATP, that does not require hydrolysis to ADP was also shown to activate RBOH proteins in Arabidopsis [39] and may also function as a signal in the autopropagation of the oxidative burst. In parallel with the apoplastic ROS wave, cytosolic H_2O_2 derived from RBOH activation could also rapidly diffuse through the symplast via plasmodesmata. An additional route for ROS travel could also be the phloem or xylem with their bordering cells that would enable the ROS signal to travel rapidly long distances up and down through the plant vasculature.

ROS long distance propagation may act in concert with other systemic signals such as jasmonic acid (JA), ethylene, methyl salicylate or electric signals that also function in cell-to-cell communication [40,41]. Recent measurements estimate the movement of JA from a wound site to distal unwounded leaves at about 4 cm min^{-1} [42]. In addition, HL-activated SAA was recently shown to be accompanied by electrical signals [41]. Because membrane potential could be directly affected by ROS accumulation and because the rate of certain electric signals in plants matches the rate of the ROS waves reported by Miller *et al.* [1,12••] it is possible that the generation of a ROS wave affects the formation, amplitude and/or rate of the electrical signal.

Rboh expression

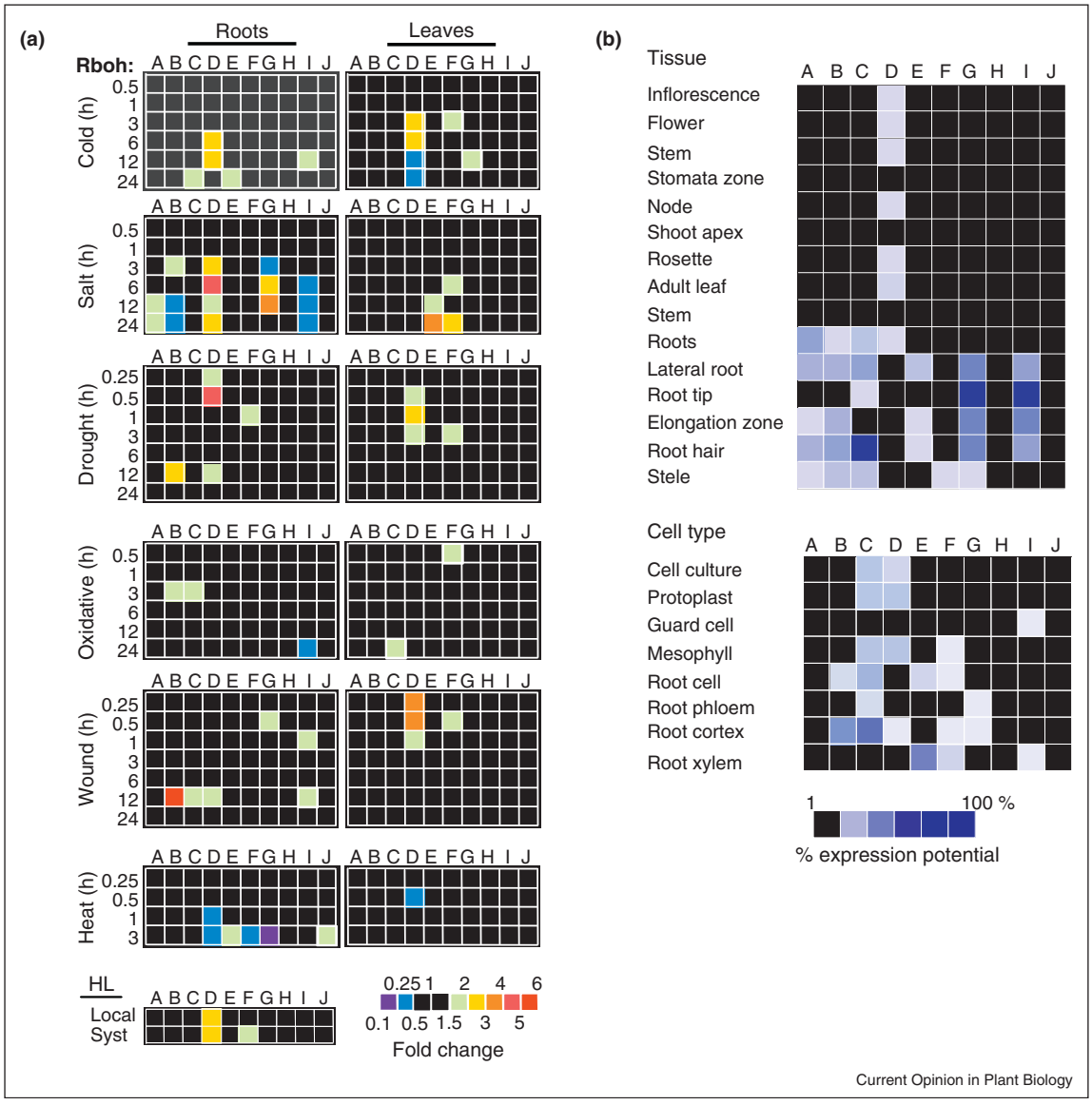
RBOH activity can be elevated by wounding, pathogens or different abiotic stress stimuli [5,7,12••,23,37,43]. Relatively little transcriptional regulation is however observed for *Rboh* genes (Figure 3). Nevertheless, *RbohD* is unique among the 10 different *Rbohs* of Arabidopsis showing a high degree of stress responsiveness both in shoots and roots (Figure 3A). Furthermore, in accordance with its role in systemic signaling and SAA response, *RbohD* is upregulated within 30 min in systemic shaded leaves in response to local HL intensity ([44]; Figure 3A lower panel), corresponding with previous observations that *RbohD* provides amplification to the HL stress-activated signaling pathways [43]. *RbohD* is expressed not only in shoot tissue but also in roots. Together with other RBOH proteins such as A–C, G and I it may form a vertical continuum of oxidative burst potential from one tip of the plant to the other.

The relative differential expression of *Rbohs* in different parts of the plant, as well as in different tissues and cell types, suggests a high degree of specialization that appears to stem from the co-expression signature of different *Rboh* genes, rather than the specific expression of a particular one, in specific cells or tissues (Figure 3B).

Integration of ROS signaling in cells

Depending on the type of ROS and its production/scavenging site, ROS can regulate a broad range of biological processes [1,4,45]. Comprehensive screening of a genome-wide mutant collection in yeast demonstrated that very specific gene sets are required to protect

Figure 3



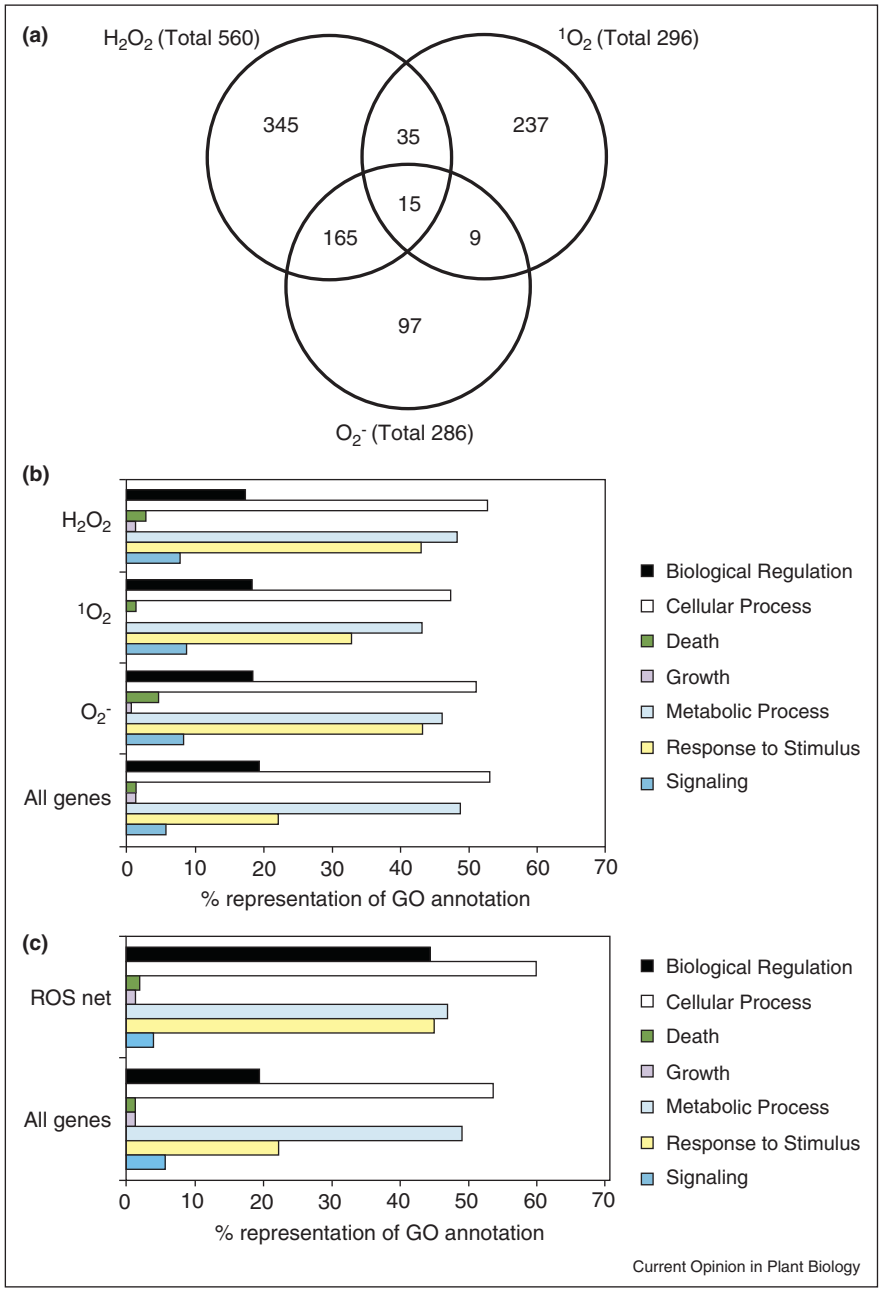
Expression pattern of the *Arabidopsis thaliana Rboh* gene family (10 *Rbohs*; *Rboh A-J*). Changes in steady-state transcript levels for all plant *Rbohs* were obtained from Genevestigator microarray database using the Meta-Profile Analysis tool [61].

(a) Transcriptional changes in response to different stress treatments in roots and leaves. Transcript abundance is relative to control treatment and color-coded to represent the fold change in expression relative to control treatment at each time point. Abbreviations: HL – high light. Syst – sytemic leaves, Local – local leaves.

(b) Relative expression level of *Rboh* A-J in plant tissues and organs (top panel) and in particular cell types (bottom panel). Data were obtained from Genevestigator microarray database using the Meta-Profile Analysis tool, Anatomy profile [61].

cells against different types of ROS [46]. Specificity in the response of plants to different types of ROS can be addressed in plants by transcriptome analyses [47–49]. A comparison of transcripts upregulated in response to different types of ROS demonstrates that out of 286 O_2^- (the ROS directly produced by RBOHs)-responsive transcripts [49], 180 transcripts (approximately 63%, Figure 4A) overlap with H_2O_2 responsive transcripts [47]. It is relatively easy to understand why O_2^- and H_2O_2 signaling function in the same cascade(s), because most of the O_2^- generated in cells dismutases to H_2O_2 spontaneously or catalytically via the function of superoxide dismutase (SOD) [50]. Nevertheless, 345 or 97 transcripts are upregulated specifically by H_2O_2 or O_2^-, respectively, indicating the existence of distinct signaling pathways activated by these different ROS. Out of 296 $^1O_2$ responsive transcripts [48], only 50 or 24 transcripts (approximately 21% or 10%, respectively) showed overlap

Figure 4



Expression of ROS response transcripts or ROS network genes in Arabidopsis.
(a) Venn diagram showing the number of transcripts significantly enhanced in Arabidopsis in response to treatment with H_2O_2 [47], O_2^- [49] or $^1O_2$ [48]. **(b and c)** Functional categorization of ROS responsive transcripts **(b)** or ROS network genes **(c)**. Genes that belong to each gene ontology (GO) category were identified using TAIR (http://www.arabidopsis.org/servlets/Search?action=new_search&type=keyword). Representation of each GO annotation category in the different gene groups was determined in ROS responsive transcripts [47–49] or ROS network genes [4]. 'All genes' indicates all identified genes in the Arabidopsis genome. ROS net indicates ROS network genes [4].

with H_2O_2 or O_2^- responsive transcripts, respectively, suggesting that $^1O_2$ is mainly involved in the activation of specific signals.

In spite of the apparent specificity in ROS signaling, revealed by transcriptome footprints (Figure 4A), representation of functional categories among the different ROS-response transcripts is similar between different types of ROS (Figure 4B). This could suggest integration of different ROS signals into the same biological processes. Such integration might be attributed at least partly to mechanisms that regulate general

responses to oxidative stress [48] or transcripts commonly activated in response to different ROS (Figure 4A). Different abiotic stresses can provoke different ROS signatures [48], and integration of ROS signals generated in response to different stresses, or at different cellular components, have been the focus of recent attention [51,52]. Another possibility is that different ROS signals regulate the same biological processes but via different pathways.

One of the main functions of ROS signaling is the regulation of defence mechanisms against biotic threats and/or acclimation to abiotic stress conditions [4,53]. In accordance, the annotation 'Response to Stimulus' is approximately 10–20% more represented in ROS-response transcripts (H_2O_2, $^1O_2$ or O_2^- [47–49]; Figure 4B) or ROS network genes (ROS net [4]; Figure 4C) compared with all genes in Arabidopsis (Figure 4B,C). Interestingly, the annotation 'Biological Regulation' is specifically over represented in ROS network genes compared with ROS-response transcripts or all genes in Arabidopsis (Figure 4B,C). This observation suggests that, compared to ROS-response transcripts, ROS network genes, that regulate cellular ROS levels [4], are involved in many different biological processes to balance cellular homeostasis both under controlled or stressed conditions. Indeed, previous studies demonstrated the role of ROS generating/scavenging systems in the regulation of growth and development under controlled growth conditions [37,54–56], as well as in response to stress [4,37,57]. The ROS network, that includes all 10 *AtRboh*s [4], is therefore a key regulatory network that controls growth, development and responses to environmental conditions.

Conclusions

RBOHs regulate a large number of important processes in plants by producing ROS that function as signal transduction molecules. They are unique among other ROS producing mechanisms in plants because they integrate different signal transduction pathways such as calcium, protein phosphorylation and lipid signaling with ROS production. RBOHs were also recently found to mediate rapid long distance systemic signaling in plants functioning in the generation of an autopropagating wave of ROS that mediates systemic gene expression. Responsible for mediating local and systemic signal transduction in plants, *Rbohs* are a central driving force of ROS signaling in cells and key for the integration of many different signal transduction pathways in plants.

Acknowledgements

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- •• of outstanding interest

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