

PLANT signaling & behavior
Volume 8, Number 1, January 2013
ISSN: 1559-2324 (Print) / 1559-2324 (Online)
Taylor & Francis Group



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To cite this article: Marina Cvetkovska, Nicole A. Alber & Greg C. Vanlerberghe (2013) The signaling role of a mitochondrial superoxide burst during stress, *Plant Signaling & Behavior*, 8:1, e22749, DOI: [10.4161/psb.22749](https://doi.org/10.4161/psb.22749)

To link to this article: <https://doi.org/10.4161/psb.22749>



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Published online: 06 Dec 2012.



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The signaling role of a mitochondrial superoxide burst during stress

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Keywords: alternative oxidase, biotic stress, manganese superoxide dismutase, mitochondrial electron transport chain, nitric oxide, organelle signaling, salicylic acid, superoxide burst

Submitted: 11/01/12

Accepted: 11/01/12

<http://dx.doi.org/10.4161/psb.22749>

Citation: Cvetkovska M, Alber N, Vanlerberghe G. The signaling role of a mitochondrial superoxide burst during stress. *Plant Signal Behav* 2013; 8:e22749; PMID: 23221746; <http://dx.doi.org/10.4161/psb.22749>.

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Addendum to: Cvetkovska M, Vanlerberghe GC. Coordination of a mitochondrial superoxide burst during the hypersensitive response to bacterial pathogen in *Nicotiana tabacum*. *Plant Cell Environ* 2012; 35:1121–36; PMID:22211396; <http://dx.doi.org/10.1111/j.1365-3040.2011.02477.x>; and Cvetkovska M, Vanlerberghe GC. Alternative oxidase impacts the plant response to biotic stress by influencing the mitochondrial generation of reactive oxygen species. *Plant Cell Environ* 2012; In press; PMID:22978428; <http://dx.doi.org/10.1111/pce.12009>.

Plant mitochondria are proposed to act as signaling organelles in the orchestration of defense responses to biotic stress and acclimation responses to abiotic stress. However, the primary signal(s) being generated by mitochondria and then interpreted by the cell are largely unknown. Recently, we showed that mitochondria generate a sustained burst of superoxide (O_2^-) during particular plant-pathogen interactions. This O_2^- burst appears to be controlled by mitochondrial components that influence rates of O_2^- generation and scavenging within the organelle. The O_2^- burst appears to influence downstream processes such as the hypersensitive response, indicating that it could represent an important mitochondrial signal in support of plant stress responses. The findings generate many interesting questions regarding the upstream factors required to generate the O_2^- burst, the mitochondrial events that occur in support of and in parallel with this burst and the downstream events that respond to this burst.

Plant mitochondria play a central role in carbon and energy metabolism, but have also been suggested to generate “signals” that influence processes such as nuclear gene expression and resistance to biotic and abiotic stress.^{1–6} Certainly, mitochondria must be capable of retrograde regulation, whereby their function generates signals able to control the expression of nuclear genes encoding mitochondrial proteins.^{7,8} However, the primary signals responsible for these phenomenon remain largely unknown, and there is only a fragmented knowledge of any signal transduction pathways or cellular processes under mitochondrial influence.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have become increasingly recognized as signal molecules involved in controlling a range of plant processes.^{9–12} The signaling function of these reactive molecules presumably requires specific mechanisms to control their synthesis, degradation and localization, all in response to particular conditions. Our knowledge of these mechanisms is generally quite limited, although substantial progress has been made in some plant systems.^{13–16}

ROS and RNS Generation by Plant Mitochondria

Mitochondria are a source of ROS. This is due to “single electron leak” from electron transport chain (ETC) components to O_2 producing superoxide (O_2^-). In both plants and animals, complexes I and III are proposed to be major sites of such electron leak^{17,18} (Fig. 1). Once produced, matrix O_2^- can be further converted to H_2O_2 by a matrix-localized manganese superoxide dismutase (MnSOD).¹⁹ The generation of RNS has also been linked to mitochondria although, in this case, the mechanism and site(s) of generation are less well understood, particularly in plants.^{18,20}

The rate of ROS generation by mitochondria depends upon the reduction state of ETC components. In animals, this reduction state is generally dependent upon the rate of electron transport and the membrane potential, which in turn are primarily dependent upon the rate of dissipation of membrane potential, particularly by oxidative phosphorylation. Hence, when ADP is readily available and being actively phosphorylated to ATP, dissipation of the proton gradient lowers

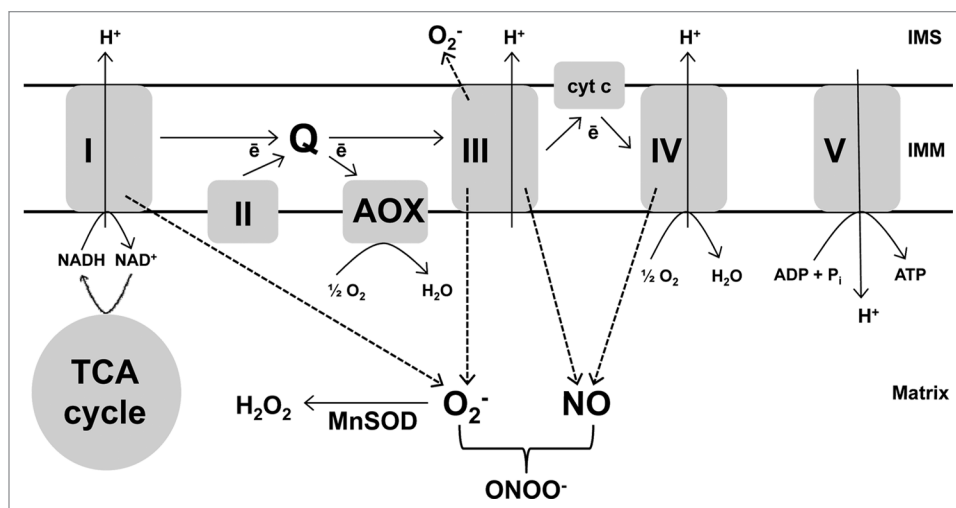


Figure 1. The plant mitochondrial ETC includes two terminal oxidases able to catalyze the 4-electron reduction of O_2 to H_2O , the usual cyt oxidase (complex IV) and AOX. Electron transport from the ubiquinone pool (Q) to complex IV is coupled to the generation of a membrane potential that is subsequently dissipated by ATP synthase (complex V) to produce ATP. However, electron flow from Q to AOX is non-energy conserving. When the ability of an ETC component to transport electrons is reduced and/or membrane potential is high, electron transport can slow, leading to an over-reduction of the ETC. Under these conditions, single electron leak to O_2 or nitrite increases, producing O_2^- and NO, respectively. In plants, the specific sites and mechanisms of O_2^- and NO generation are not yet well understood. See text for further details. I, II, III, IV, V: complexes I to V.

membrane potential and O_2^- generation is less than when ADP is limiting. In plants, however, the relationship between electron transport, oxidative phosphorylation and ROS generation is more complex due to the presence of alternative oxidase (AOX), an additional ETC component (Fig. 1). AOX catalyzes the oxidation of ubiquinol and reduction of O_2 to H_2O , effectively acting as a branch in the ETC whereby electron flow bypasses proton-pumping complex III, cytochrome (cyt) c and proton-pumping complex IV (cyt oxidase).²¹ Significantly, electron flow from ubiquinol to AOX is not proton-pumping and hence does not contribute to membrane potential (Fig. 1). Hence, AOX could provide a means to maintain electron flow while still preventing the over-reduction of the ETC.

Using fluorescent confocal microscopy, we recently showed that tobacco leaves with suppressed levels of AOX, due to RNA interference, have increased concentrations of O_2^- in their mitochondria.²² This provides direct in planta evidence that one function of AOX is to prevent the over-reduction of ETC components that leads to single electron leak. Interestingly, the same study also found higher levels of nitric oxide (NO), a RNS, in the leaves lacking AOX and this appeared to be due, at least in part, to higher levels of NO in mitochondria. One means by

which mitochondria may generate NO is via single electron leak from the ETC to nitrite.^{18,20} Hence our results suggest that AOX, by controlling the reduction state of the ETC, also dampens the generation of NO. Our conclusions were further supported by experiments with antimycin A (AA), a complex III inhibitor that, by restricting electron flow, causes an over-reduction of ETC components. In wild-type plants, both mitochondrial O_2^- and NO increased dramatically in response to AA. However, these increases were not seen in plants overexpressing AOX and hence able to maintain high rates of electron flow, even in the presence of AA.²³

Plant Mitochondria as Signaling Organelles During Biotic Stress

We hypothesized that plants may use mitochondrial-derived ROS and RNS as signaling molecules, and further that AOX could provide a means to modulate their generation, particularly during stress.²⁴ Below we describe recent evidence supporting this hypothesis.

It is well known that plant infection by a pathogen can lead to the rapid activation of a plasma membrane-localized NADPH oxidase.^{10,14} This activation results in an apoplastic “oxidative burst” that is closely associated with subsequent intracellular

signaling events that culminate in defense responses such as the hypersensitive response (HR), a programmed cell death (PCD) at the site of infection.^{25,26} Beside this well-characterized apoplastic source of ROS, it is possible that intracellular sources of ROS (and RNS) are also important in orchestrating defense responses to pathogens. The mitochondrion is one such potential source that has been implicated.²⁷⁻²⁹

Recently, we examined the interaction of *Nicotiana tabacum* with the bacterial pathogen *Pseudomonas syringae*.³⁰ We showed that the incompatible *P. syringae* pv *maculicola* induced defense responses that included the HR, which was preceded by an early and persistent increase of O_2^- in the mitochondrial matrix (Fig. 2). This “ O_2^- burst” was specific to the interaction with the HR-inducing pv *maculicola*, not being seen in response to a compatible (i.e., disease-causing) pv or in response to the incompatible pv *phaseolicola* that induced well-known defense responses but not including the HR. The disparate effect of the two incompatible pv’s appears to be due to a coordinated response of AOX (as a means to modulate the rate of O_2^- generation) and MnSOD (the sole enzymatic means to scavenge matrix O_2^-). While pv *phaseolicola* infection resulted in a strong induction of AOX and a maintenance

of high MnSOD activity, *pv maculicola* infection failed to induce AOX and was accompanied by a loss of MnSOD activity³⁰ (Fig. 2). We further established that, in transgenic AOX knockdown plants unable to induce AOX in response to *pv phaseolicola*, a O_2^- burst was now generated in response to infection.²³ Further, AOX knockdown plants infected with *pv maculicola* displayed a delayed O_2^- burst that manifested itself in a delayed HR. These results place AOX as a potential key regulator of a mitochondrial O_2^- -based signaling pathway that subsequently impacts plant responses to biotic stress.^{23,30}

It is worth emphasizing that the O_2^- burst we have reported is clearly distinct from “mitochondrial O_2^- flashes” that have been detected, mostly in animals but also in plants.^{31,32} Those apparent flashes in O_2^- are short-term (seconds) in duration and have been detected using circularly permuted yellow fluorescent protein as the O_2^- sensor. Recently, it has been strongly argued that these O_2^- flashes are in fact an artifact of the detection system.³³ On the other hand, the O_2^- bursts we have reported are long-lived (hours) and have been measured using a mitochondrion-localizing version of hydroethidine (MitoSOX; Molecular Probes), a well-established small-molecule fluorescent O_2^- probe.^{23,30} As controls, we showed that the fluorescence signal observed with this probe could be strongly attenuated by the O_2^- scavenger SOD-PEG (a membrane-permeable SOD) or strongly amplified by AA.

Characterization and Significance of the Mitochondrial O_2^- Burst

Our results^{23,30} generate many interesting questions about mitochondrial signaling in general and the O_2^- burst, in particular. Some of these questions are summarized in Figure 3 and further discussed below.

First, work to date has been done exclusively with *P. syringae*. Hence, it will be interesting to establish whether the HR induced by viruses and fungi is also preceded by a O_2^- burst. Changes in mitochondrial ROS have been implicated as important during such pathogen interactions.^{3,4,34,35} Further, AOX overexpression did result in smaller TMV-induced HR

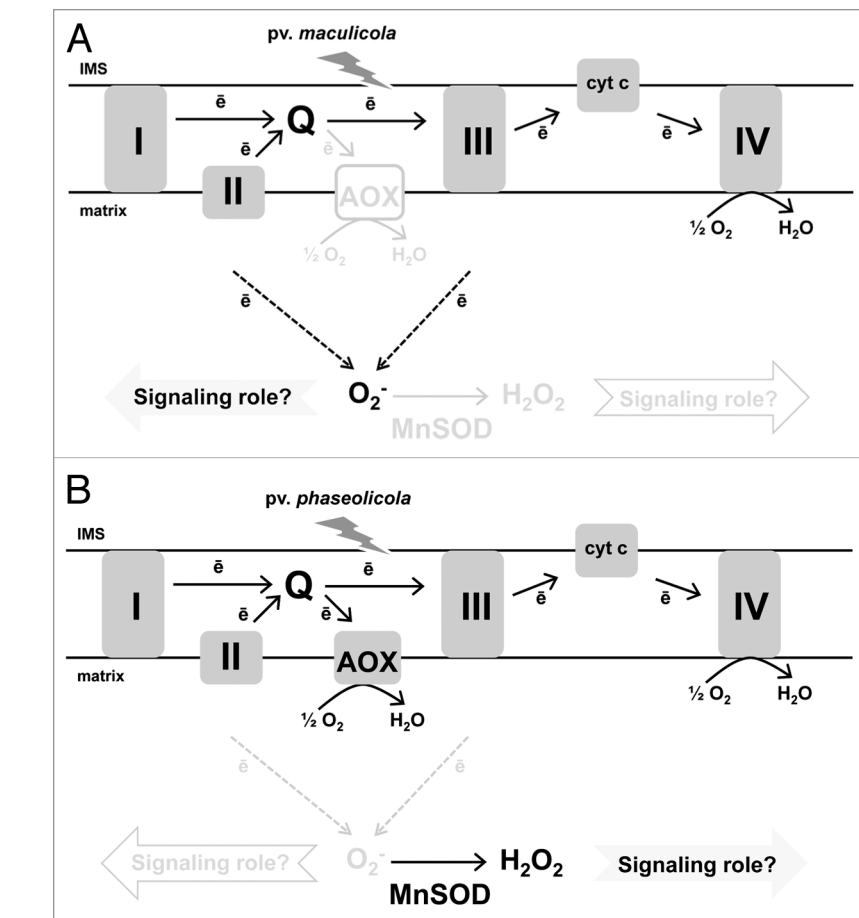


Figure 2. The impact of two incompatible *pv*'s of *P. syringae* on the mitochondria of tobacco leaf mesophyll cells. (A) Infection with the HR-inducing *pv maculicola* results in an early and persistent burst of O_2^- in the mitochondrial matrix that may have a signaling role in support of the HR. (B) Infection with *pv phaseolicola*, that causes induction of plant defenses but not including the HR, lacks a matrix O_2^- burst. The differential effect of the two *pv*'s is supported by a coordinated response of the major ETC mechanism to avoid O_2^- generation (AOX) and the sole enzymatic means to scavenge matrix O_2^- (MnSOD). In response to *pv phaseolicola*, AOX is strongly induced and MnSOD activity remains high, while in response to *pv maculicola* MnSOD activity declines and AOX remains low. As a result, the two bacterial *pv*'s each generate distinct mitochondrial ROS signatures that may impact defense responses and cell fate.

lesions.³⁶ However, definitive experiments to test for the appearance of a O_2^- burst in response to such pathogens are now needed.

If the mitochondrion has a signaling role during biotic stress, it would be interesting to establish whether this role extends to abiotic stresses as well. A common theme among several disparate mitochondrial mutants is their increased or decreased stress tolerance.^{2,4,37,38} Are specific mitochondrial ROS and/or RNS signatures responsible for this altered tolerance state? Disparate mitochondrial mutants also exhibit changes in stomatal aperture, particularly under stress conditions.³⁹⁻⁴¹ An intriguing possibility is that

changes in mitochondrial-derived ROS and/or RNS are impacting the signal path(s) that control stomatal movement.

Previous work shows that AOX can provide a level of protection against PCD.²⁴ Since the HR is a natural example of PCD that is likely of benefit to the plant, it is perhaps not surprising that AOX would be kept suppressed after *pv maculicola* infection. An interesting question is how this suppression is achieved since infection results in the rapid elevation of several molecules previously described as inducers of AOX synthesis such as salicylic acid (SA), NO and H_2O_2 . We provided evidence that SA levels above a threshold amount might be responsible suppressing

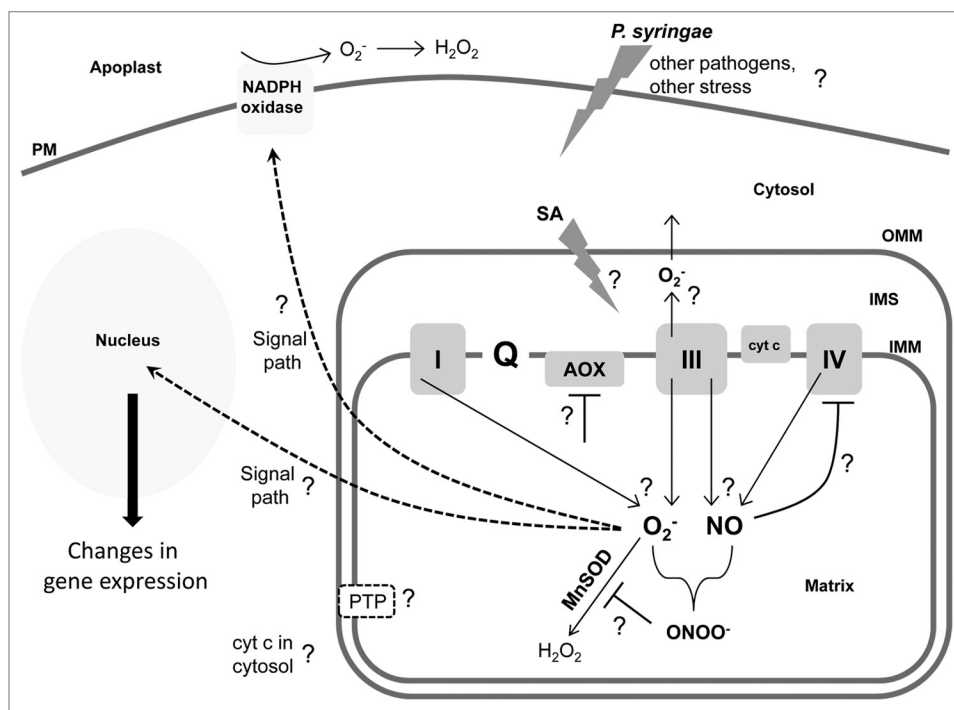


Figure 3. Simple cartoon of a plant cell and mitochondrion. This figure is meant to highlight some key questions regarding the upstream factors required to generate a mitochondrial O_2^- burst, the mitochondrial events that occur in support of or in parallel with this burst, and the downstream events that may be responsive to this burst. See text for further discussion of these aspects. PM, plasma membrane; OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane.

AOX induction, but this is clearly an area that requires further study. Whether AOX activity is also being suppressed by some post-translational mechanism is also relevant. In this regard, it is intriguing that AOX has been identified as a target of tyrosine nitration, albeit the study was not in relation to biotic stress and how the modification impacted AOX activity is not known.⁴² Suppression of AOX activity could perhaps also occur by oxidation of regulatory cysteine thiols⁴³ but again, whether this occurs in response to pathogen is not yet known.

As discussed earlier, membrane potential is typically a key factor determining the rate of O_2^- , and perhaps NO, generation by the ETC. A persistent O_2^- burst implies that membrane potential remains high and perhaps even increases after infection with the HR-inducing pv *maculicola*. A few studies have reported increases in membrane potential as an early event preceding various sorts of plant PCD, but more often it has been reported that loss of membrane potential is an early event.⁴⁴ Loss of membrane potential is sometimes attributed to the opening

of a permeability transition pore (PTP), which in turn is often implicated to result in the release of cyt c to the cytosol. Unfortunately, the cause-effect relationship of these mitochondrial events, how they relate to O_2^- generation and how they impact cell fate is still largely unknown and is an important area for continued study.⁴⁴ One possible means to promote a high membrane potential, beside keeping AOX activity low, would be disabling of the adenine nucleotide translocator, which could restrict oxidative phosphorylation and hence dissipation of membrane potential.⁴⁵

While AOX knockdown plants have constitutive higher amounts of O_2^- than wild-type, this amount is nonetheless further strongly enhanced by the interaction with incompatible *P. syringae* indicating that, in addition to keeping AOX low, additional factor(s) associated with infection are required for the O_2^- burst to fully manifest itself. These factors are unknown but a prime candidate may be SA. Studies with plant suspension cells and isolated mitochondria strongly suggest that SA can target mitochondrial function.⁴⁶ However,

its mechanism and site of action are not well understood, and the reported effects of SA on energy metabolism need further confirmation in intact leaf. Interestingly, our results hinted that complex III might be the mitochondrial target of biotic stress,²³ and studies with isolated mitochondria have implicated SA to impact the function of this complex.⁴⁷ The mode of action of SA on mitochondria may also differ depending upon SA concentration.⁴⁶ This provides a potential mechanism by which the different incompatible pv's, which did elicit different SA amounts,³⁰ could have impacted mitochondria in disparate ways. Besides SA acting on the mitochondrion, another possibility is that increased NO inhibits cyt oxidase, which could then promote further generation of NO and O_2^- due to over-reduction of the ETC. NO is a potent inhibitor of cyt oxidase, but AOX is NO-resistant,⁴⁸ which further illustrates how AOX amount could strongly impact mitochondrial function during biotic stress.

Beside suppressing AOX induction, pv *maculicola* infection was associated with a loss of MnSOD activity, which likely

supported the O_2^- burst by lessening the conversion of O_2^- to H_2O_2 in the matrix. The mechanism by which MnSOD activity is lost is not known. Interestingly, animal MnSOD is a major target of tyrosine nitration, resulting in a loss of activity.⁴⁹ Tyrosine nitration requires peroxyntirite ($ONOO^-$), the product of the reaction of O_2^- and NO (Fig. 1). As MnSOD activity is lost, it promotes still higher O_2^- , which in turn promotes further $ONOO^-$ formation (if NO is available) and further MnSOD inactivation. It would be interesting to see whether a similar vicious circle is responsible for the loss of MnSOD after *pv maculicola* infection, which certainly was associated with increased levels of $ONOO^-$.²³

Another important area for future research will be to establish the site(s) and topology of O_2^- generation by the ETC, particularly in relation to it acting as a signal molecule. For example, while complex I is thought to release O_2^- exclusively to the matrix side of the inner mitochondrial membrane (IMM), complex III may release O_2^- to both sides of the IMM (Fig. 1). Further studies should establish whether the matrix O_2^- burst measured by MitoSOX in our experiments was accompanied by additional O_2^- release to the inner membrane space (IMS).

Several studies have linked specific mitochondrial events with subsequent downstream changes in gene expression.⁴⁴ For example, Maxwell et al.¹ identified a group of typically biotic stress-responsive genes whose expression in response to mitochondrial dysfunction could be blocked by bongkreic acid, an inhibitor of the PTP. Collectively, such studies provide a list of candidate genes that may be responsive to a mitochondrial O_2^- burst. Plants with modified AOX expression provide a means to manipulate the O_2^- burst and hence will be a useful tool to link the burst with specific gene expression profiles.

Another interesting area of study would be to establish the relationship between the mitochondrial O_2^- burst and the oxidative burst generated in the apoplast. As expected, we found that the HR-inducing *pv maculicola* generated a typical oxidative burst (measured as whole leaf H_2O_2) that was not seen with *pv phaseolicola*. However, when we elicited a mitochondrial O_2^- burst

in *pv phaseolicola* using the AOX knock-down plants, this was now accompanied by a large and sustained increase in whole leaf H_2O_2 .²³ While this could perhaps be explained by the mitochondrial O_2^- burst providing all the substrate for this H_2O_2 rise, another intriguing possibility is that the mitochondrial O_2^- burst lead to activation of NADPH oxidase. In animals, there are several model systems in which activation of NADPH oxidase depends upon intracellular ROS.⁵⁰ Interestingly, it is reported in tobacco that SA potentiates the oxidative burst⁵¹ and that intracellular ROS rises prior to the oxidative burst.²⁹

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

G.C.V. is grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC) and the University of Toronto Scarborough for their support of his research program on the alternative oxidase of plant mitochondria.

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