The Roles of Mitochondrial Reactive Oxygen Species in Cellular Signaling and Stress Response in Plants[1][OPEN]

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Mitochondria produce ATP via respiratory oxidation of organic acids and transfer of electrons to O_2 via the mitochondrial electron transport chain. This process produces reactive oxygen species (ROS) at various rates that can impact respiratory and cellular function, affecting a variety of signaling processes in the cell. Roles in redox signaling, retrograde signaling, plant hormone action, programmed cell death, and defense against pathogens have been attributed to ROS generated in plant mitochondria (mtROS). The shortcomings of the black box-idea of mtROS are discussed in the context of mechanistic considerations and the measurement of mtROS. The overall aim of this update is to better define our current understanding of mtROS and appraise their potential influence on cellular function in plants. Furthermore, directions for future research are provided, along with suggestions to increase reliability of mtROS measurements.

GENERATION OF MITOCHONDRIAL ROS

Molecules typically referred to as reactive oxygen species (ROS) in plant cells include ozone, singlet oxygen, superoxide, H_2O_2, and the hydroxyl radical. There is no reliable information on any significant generation of ozone and singlet oxygen by plant mitochondria, and the short half-life of the hydroxyl radical makes it incompatible with specific roles in signal transduction through selective modification of target molecules. This leaves superoxide and H_2O_2 as good candidates for ROS generated in plant mitochondria (mtROS) of regulatory significance. Superoxide is formed through single electron reduction of O_2, and the respiratory Complexes I, II, and III have all been identified as major production sites but display fundamentally different rates and topologies of superoxide release (Murphy, 2009). The relative physiological contributions of each also remain notoriously hard to dissect. Plant-focused studies have provided some mechanistic insight (Rich and Bonner Jr, 1978; Møller, 2001; Gleason et al., 2011; Jardim-Messeder et al., 2015), but the bulk of the evidence is still derived from more detailed mammalian studies (Murphy, 2009). While superoxide production occurs during normal operation of the respiratory chain, its rate is strongly increased when respiratory rate is slowed down, e.g. by restricted ADP availability or respiratory chain inhibition, leading to a highly reduced state of mitochondrial electron transport chain (mtETC) components (Møller, 2001).

Superoxide production by the mtETC can be minimized by a variety of pathways in plant mitochondria that bypass the classical mtETC and oxidative phosphorylation. Uncoupling proteins promote proton leak across the membrane (Sweetlove et al., 2006), while NDs and AOXs bypass proton pumping by electron flow via Complex I, and Complex III and IV, respectively. The role of AOX in minimizing ROS production has been extensively reviewed (Rhoads et al., 2006; Vanlerberghe, 2013). Recently, overexpression of the Arabidopsis (*Arabidopsis thaliana*) uncoupler protein Uncoupling protein1 in tobacco was found to reduce the rate of ROS generation, induce the antioxidant defense system, and enhance resistance to multiple abiotic stresses (Barreto et al., 2014). Superoxide spontaneously disproportionates to H_2O_2 and O_2, but this can be strongly accelerated by superoxide dismutase (SOD; Møller, 2001; Morgan et al., 2008). Alternatively, superoxide readily

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reacts with other radicals such as NO or redox-active metal centers of other proteins (Tan et al., 2010). These reactions outcompete interactions with abundant biomolecules such as the amino acid residues of proteins, nucleic acids, lipids, and sugars. Suppression of the mitochondrial matrix manganese SOD in Arabidopsis disturbs thiol redox homeostasis of the matrix, retards root growth (Morgan et al., 2008), and leads to disruption of female gametogenesis (Martin et al., 2013). The H_2O_2 released by the matrix SOD can either be removed on site or exit the matrix. In both cases, efficient detoxification systems are in place and selective oxidation of regulatory targets may relay information on mitochondrial processes.

ROS SIGNALING WITHIN MITOCHONDRIA BY THIOL SWITCHING?

Specific protein thiols may be oxidized by H_2O_2. Several studies have found redox-active thiols on matrix proteins, some of which have been shown to adjust protein function in vitro, raising the possibility of intramitochondrial ROS-dependent thiol redox signaling to adjust functions at the protein level. Examples that have been specifically characterized include AOX (Yoshida et al., 2013) and several tricarboxylic acid (TCA)-cycle enzymes (Schmidtmann et al., 2014; Yoshida and Hisabori, 2014; Daloso et al., 2015), with the TRX system as the likely endogenous reductant. Gel-based redox proteomics and TRX-trapping approaches have identified even more mitochondrial proteins that can, in principle, undergo thiol switching (Winger et al., 2007; Yoshida et al., 2013). For all of these proteins, it remains unresolved if the respective thiol switch can be oxidized by mtROS (directly or indirectly) and maintained in its oxidized state, considering the presence of a highly efficient rereduction system under physiological conditions. A switch would be a prerequisite for bona fide redox-regulation, as opposed to TRX-mediated 'maintenance' of a thiol in its reduced state. A particularly intriguing example of intramitochondrial thiol redox regulation has been studied in mammals where Complex I can be deactivated by reversible oxidation of a Cys-39 of ND3 (Chouchani et al., 2013) to avoid excessive superoxide release from reverse electron flow at reoxygenation after anoxia. The Cys motif at ND3 is well conserved in plants (Cys-45 in Arabidopsis; Braun et al., 2014), but evidence for reverse electron flow is currently lacking in plants. However, given the conservation it appears worthwhile to investigate its potential plant function, such as during the recovery from water-logging.

MEASURING MtROS IN VITRO AND IN VIVO

The generation of mtROS is routinely studied using colorimetric and fluorescent detection systems. Probes such as 3,3′-diaminobezidine, nitroblue tetrazolium (NBT), AmplexRed (for isolated mitochondria), 2′,7′-dichlorofluorescein derivatives, and hydroethidine have been reported to detect or even quantify H_2O_2, superoxide, or mtROS in plant studies (Gleason et al., 2011; Li and Xing, 2011; Martin et al., 2013). However, there are legitimate concerns about their specificity and subcellular accumulation and their interference with endogenous redox biology (Bonini et al., 2006; Zielonka and Kalyanaraman, 2010; Miwa et al., 2016), prompting the search for straightforward and applicable alternatives. Probes that can be specifically placed in mitochondria, either through electrical potential-driven accumulation (e.g. MitoSOX Red; Miller et al., 2009; Li and Xing, 2011; Martin et al., 2013; Liu et al., 2014) or through genetic targeting for fluorescent protein sensors (Schwarzländer et al., 2016) have addressed the localization issue. However, their chemical specificity in mitochondria also remains a concern. For example, cpYFP that was reported as responsive to superoxide and has been used as such also in plant mitochondria (He et al., 2012) has been conclusively shown not to react to superoxide, but to pH changes (Schwarzländer et al., 2014), while the H_2O_2-responsive HyPer family of fluorescent protein probes (Costa et al., 2010) also suffer from pH artifacts, making measurements problematic in the matrix where pH can change rapidly (Schwarzländer et al., 2012b). Dynamic measurements using such probes also rely on efficient probe regeneration after oxidation via the endogenous glutathione/glutaredoxin system. Yet, the only known matrix GRX (GRXS15) shows very low thiol exchange activity (Moseler et al., 2015; Stroeher et al., 2016). Rigorous application and further optimization of HyPer probes as well as peroxidase-coupled, pH-insensitive roGFP sensors, like roGFP2-Orp1 (Gutscher et al., 2009), appear to offer the best potential to allow specific measurements of a defined mtROS (H_2O_2) and allow physiological interpretation. This may be complemented by mass spectrometry-based approaches that have been developed in mammalian mitochondria (Cochemé et al., 2011), but have not yet been used in plants.

Given the persisting methodological constraints, it is important to note that most historical reports of mtROS production in planta are only one interpretation of the chosen monitoring technique (i.e. chemical reaction product, optical probe, marker metabolite, or marker transcript) and are often based on assumptions that may or may not be justified in a particular case as discussed above. Future experiments should involve a transparent appraisal of the chosen technique or cellular mtROS indicator, which will allow accumulating evidence from orthogonal approaches across studies to paint a clearer picture.

CONSIDERATIONS FOR TRANSDUCTION OF MtROS SIGNALS OUT TO THE CELL

Movement of mtROS signals out of mitochondria requires a direct and sensitive target and a means of transducing the signals from the various intramitochondrial locations to the cytosol. If there is a direct downstream target of a mitochondrial superoxide

signal, proteins with transition metal cofactors are likely to be the first candidates. An intriguing mechanism has been proposed in yeast and mammals by which the activity of cytosolic SOD1 controls mitochondrial respiration (Reddi and Culotta, 2013), but plants have not yet been investigated for comparable signaling. Efficient dismutation of superoxide inside mitochondria, however, makes H_2O_2 a more likely signal, aided by its much longer estimated lifetime and the ability for channel-mediated membrane passage through aquaporins (Bienert and Chaumont, 2014). At least one aquaporin (TIP5;1) can localize to the mitochondria in Arabidopsis (Soto et al., 2010). However, the reactivity of H_2O_2 is also remarkably limited by kinetic competition and most thiols are not particularly reactive for H_2O_2 within the cellular environment in the absence of catalysis. Exceptions, however, can be found in thiols of a small group of proteins that have been found enriched in mitochondria (Riemer et al., 2015). Particularly interesting are the thiol peroxidases, which have been recognized as among the most H_2O_2-reactive thiols in cells (Perkins et al., 2015) and are key players in H_2O_2 detoxification in plants (Dietz, 2011). With PrxII F and a GPX-like protein, two thiol peroxidases have so far been found in plant mitochondria (Finkemeier et al., 2005; Navrot et al., 2006). Low K_d and high reactivity of the catalytic peroxiredoxin thiols can generate specific H_2O_2 drains that outcompete other thiols and ensure that a large part of H_2O_2 turnover flux will be focused to one specific thiol (Veal et al., 2007). This would provide kinetic control of H_2O_2 dynamics in vivo keeping ambient H_2O_2 levels low and protecting other biomolecules from oxidation. To serve as a signal, this information must be conserved, perhaps by dividing regeneration into a rapid-turnover, high-flux antioxidant pathway and a slow-turnover, low-flux signaling pathway, with their relative contributions set by distinct biochemical properties. Once translated, the H_2O_2 signal could then regulate downstream protein targets by a thiol switch or be further transduced by thiol-redox signaling (Riemer et al., 2015). This provides a means to quantify H_2O_2 flux by 'counting' molecules detoxified at a specific location. Maintaining information on the location of H_2O_2 sensing appears critical to discriminate between stimuli from distinct sources and to mount specific responses. Oxidation of mitochondrial proteins followed by proteolysis and export of the peptides has been proposed as a scenario of this general concept (Møller and Sweetlove, 2010). Indeed, oxidative modification has been observed for plant mitochondrial proteins (Kristensen et al., 2004; Tan et al., 2010), but further work will be required to clarify to what extent this occurs under physiological conditions in vivo.

Increasing experimental evidence supports the concept of colocalization of ROS signal and ROS sensing. Recently, mtETC-derived H_2O_2 were shown to be perceived in the intermembrane space (IMS) triggering a phosphorylation cascade in animals (Patterson et al., 2015). As the interface between the mitochondrion and the rest of the cell, the IMS hosts its own redox machinery, which may make it well suited as the integration site to prepare mtETC-derived ROS signals for transduction across the cell before they get quenched by the strong antioxidant defenses of the cytosol. Placing the ROS-sensing system close to the mitochondrial surface, where H_2O_2 is thought to pass the outer mitochondrial membrane (OMM) via porins offers similar properties. Contact sites between endoplasmic reticulum (ER) and mitochondria may act as such hotspots (Jaipargas et al., 2015; Mueller and Reski, 2015). Although the exact mechanism remains to be determined, thiol switch-based sensing of mitochondrial-derived H_2O_2 at ER contact sites makes an attractive hypothesis. The reverse, i.e. H_2O_2 produced by the ER to be sensed within the mitochondrial matrix inducing glutathionylation of a Gly decarboxylase subunit, has been proposed recently (Hoffmann et al., 2013). A recent study suggests that the twin-Cys proteins At12cys1/2 can relocate from the mitochondria to cytosol and chloroplast upon Complex I dysfunction, providing a potential mechanism to signal mitochondrial dysfunction to the rest of the cell in plants (Wang et al., 2016).

MtROS AND RETROGRADE SIGNALING

Mitochondria can signal information about their functional status to the nucleus, leading to targeted transcriptional responses, a process often described as mitochondrial retrograde regulation (MRR; Rhoads and Subbaiah, 2007; Ng et al., 2014). Many factors involved in the transcriptional regulation of MRR have been identified and belong to a range of functional classes, including transcription factors (NAC, WRKY, ABI4) and cyclin-dependent kinases (Ng et al., 2014). Also, an antagonistic relationship between auxin and retrograde signaling has been shown in a variety of mutant backgrounds (including PIN1, BIG, and AS1; Ivanova et al., 2014). Surprisingly, MRR mediators ANAC013 and ANAC017 are bound to the ER (De Clercq et al., 2013; Ng et al., 2013). Upon stress, they are thought to be released and relocate to the nucleus to exert transcriptional regulation. This identified the ER as a key signaling intermediate in MRR, and based on inhibitor studies, the proteolytic release of NAC transcription factors (TFs) may be mediated by rhomboid-class proteases (Ng et al., 2013).

The identity of the signal(s) that relays information from a dysfunctional mitochondrion via the ER to the downstream transcription factor cascades currently remains unknown. Evidence so far suggests that TCA cycle intermediates like citrate have relatively mild transcriptional effects, and TCA-intermediate signaling does not appear to occur via the NAC pathway, as the affected genes are very different (Finkemeier et al., 2013). However, several individual findings point toward a role for mtROS, and taken together they make a strong case. One of the most commonly used ways of triggering MRR in plants has been treatment with complex III inhibitor antimycin A (AA). AA treatment is well established to induce retrograde marker genes

such as *AOX1a* in Arabidopsis (Dojcinovic et al., 2005; Ng et al., 2013). AA application to plant cells induced increased ROS production as detected by 2′,7′-dichlorofluorescein accumulation in the mitochondria (Maxwell et al., 1999), suggesting that the ROS produced by AA could act as signaling intermediates. Further in line with a signaling role for mtROS, AA-induced retrograde signaling was suppressed when ROS production was reduced by overexpression of AOX in tobacco (Maxwell et al., 1999).

Other studies also indirectly suggest a correlation between mitochondrial defects, (mt)ROS formation, and retrograde signaling. For example, Complex I subunit *ndufs4* mutants have a higher basal superoxide production rate as measured by NBT staining as well as constitutive retrograde marker gene expression (Meyer et al., 2009). Other studies have looked at nuclear transcription in backgrounds with mtROS-related enzymes, such as manganese SOD *msd1* knock-down plants, and AA treatment in peroxiredoxin II F *prxII F* mutants compared to wild type (Schwarzländer et al., 2012a). However, both studies showed relatively weak transcriptional changes in common MRR marker genes, so it is difficult to draw clear conclusions for the role of mtROS in retrograde signaling from these experiments.

More evidence for a role of mtROS in retrograde signaling comes from the observation that MRR responses show remarkable overlaps with transcriptional responses to different ROS triggers and abiotic stress, e.g. H_2O_2 and salt stress. This is the case both for chemically induced MRR as well as MRR caused by genetic defects in mitochondrial components (Van Aken et al., 2007; Meyer et al., 2009; Van Aken and Whelan, 2012). Furthermore, many of the core ANAC017-dependent transcripts affected by AA also showed altered responses to H_2O_2 application (Ng et al., 2013). Remarkably, 87% of transcript changes caused by H_2O_2 were affected in *anac017* mutants. This indicates that ANAC017 is a key regulator of H_2O_2 responses and that at least one avenue for ROS production may be via mitochondria. Future work will be required to understand how mtROS signals would trigger such MRR response in a mechanistic way.

MtROS AND PLANT HORMONE SIGNALING

Plant hormones play a key role in regulating growth and development and in response to stresses. Hormone-ROS interactions have recently been reviewed (Del Río, 2015) as well as links between MRR regulation and hormone signaling (Berkowitz et al., 2016). Both connections have recently been brought together by accumulating evidence indicating that mitochondria play a role in regulation of plant hormone signaling, such as abscisic acid (ABA) and auxin, and that mtROS may be key intermediates.

Evidence for an interplay between ABA and mitochondria via mtROS has been found in lines with different sensitivity to ABA (Laluk et al., 2011; He et al., 2012; Murayama et al., 2012; Sechet et al., 2015). Collectively, these reports show that the underlying causes of increased ABA sensitivity in a range of mutants are mitochondrial defects. In some of these mutants, increases in ROS were reported, while others report decrease, using a variety of different detection methods. Given that mitochondria are crucial during early germination and seedling establishment (both are repressed by ABA; Howell et al., 2006; Wang et al., 2014), it appears conceivable that mitochondrial mutants are particularly susceptible to unfavorable germination conditions. While more evidence is required to test if mtROS have a direct role in ABA signaling, transcription of a range of genes encoding mitochondrial proteins is clearly responsive to ABA (Van Aken et al., 2009; Wang et al., 2014).

Regulation of auxin homeostasis has been linked to increased ROS levels (Tognetti et al., 2012) and mitochondria have been suggested to be involved. By screening for *more axillary shoot* mutants, mitochondrial AAA-protease *AtFtSH4* was identified. Loss of *FtSH4* function resulted in a reduction in protein stability in the mitochondria and increased H_2O_2 levels, which may be used for peroxidase-mediated oxidation of indole-3-acetic acid and cause excessive axillary branches and a dwarf phenotype (Zhang et al., 2014b). Also overexpression of mitochondrial *AtPHB3* or MRR/ROS-signaling target gene *UGT74E2* (an auxin glycosyltransferase) results in profuse shoot branching (Van Aken et al., 2007; Tognetti et al., 2010). It was previously shown that *ftsh4* mutants show induction of a number of MRR/ROS marker genes, providing further links between mtROS signaling and auxin homeostasis (Gibala et al., 2009).

Treatment with AA resulted in decreased indole-3-acetic acid levels and changes in other genes related to auxin conjugation, transportation, and receptors (Ivanova et al., 2014). In reverse, treatments or mutations that block polar auxin transport (e.g. *pin1*) result in a hyperactivated MRR response to AA (Ivanova et al., 2014; Kerchev et al., 2014). This antagonistic relationship between auxin and MRR could provide the plant with a switching mechanism between growth under optimal conditions (no MRR signals, auxin-stimulated growth) and growth-arrest during stress conditions (MRR signaling negatively affects auxin-induced growth). Furthermore, it may allow the plant to reverse MRR-induced growth arrests when the mitochondrial dysfunction has been resolved.

MtROS IN PROGRAMMED CELL DEATH AND PATHOGEN RESPONSE

mtROS production in plants has been implicated in the execution of programmed cell death (PCD; Van Aken and Van Breusegem, 2015). Great efforts have been made to validate relatively well-described yeast or animal PCD pathways in plants; yet with limited success. For instance, cytochrome *c* release, which is also observed in plants during hypersensitive response (HR)-induced PCD, may merely reflect a final phase of

mitochondrial dysfunction, rather than an initial step of the process (Yao et al., 2004). mtROS production is often increased during stress and PCD, likely due to mtETC inhibition. Several studies support this by showing that mtETC enzymes, such as AOX, protect against PCD (Li and Xing, 2011; Liu et al., 2014; Wu et al., 2015). A recent study showed that mitochondrial Complex I is responsible for the ROS production that leads to PCD in the *Arabidopsis mosaic death 1* (*mod1*) mutant, even though the MOD1 protein is active in chloroplasts (Wu et al., 2015). A critical role for mtROS, but not apoplastic or chloroplast ROS, has also been suggested in harpin-induced PCD (Garmier et al., 2007).

Mitochondria have also been proposed to be linked with PCD via increased ROS production during pathogen defense, such as during the HR (Lam et al., 2001; Mur et al., 2008; Van Aken and Van Breusegem, 2015). In agreement, a wide variety of mutants in mitochondrial functions have been reported to have altered PCD or pathogen defense phenotypes. Upon infection with the bacterial pathogen *Pseudomonas syringae* and PPIX treatment, Arabidopsis protein ACD2 (Accelerated Cell Death 2) changed its location from chloroplastic to both chloroplastic and mitochondrial (Yao and Greenberg, 2006). More recent work showed that the ACD2-substrate red chlorophyll catabolite, a toxic chlorophyll-breakdown product that can trigger ROS production, can shift to mitochondria to cause ROS-induced PCD (Pattanayak et al., 2012). This suggests that mitochondrial ROS formation may be the key cause of PCD induction in *acd2* mutants, further supported by the observation that ectopic targeting of antioxidant enzymes like ascorbate peroxidase and catalase to the mitochondria can suppress PCD (Yao and Greenberg, 2006).

Perturbations in the mtETC that are linked to ROS metabolism can result in altered pathogen susceptibility. A well-known example came from analysis of *Nicotiana sylvestris* CMSII line with complex I deficiency (Garmier et al., 2002; Dutilleul et al., 2003b). There are dramatic differences in antioxidant and defense gene expression between wild-type and CMSII leaves inoculated with a bacterial elicitor (Garmier et al., 2002). CMSII(N) plants have higher resistance to tobacco mosaic virus than wild-type plants, mainly due to the inhibition of the virus by the high level of leaf antioxidant enzymes (Dutilleul et al., 2003b). During *Erwinia amylovora* hairpin-induced HR, this CMSII line showed impaired AOX engagement, but an increase in mitochondrial SOD activity and antioxidant capacity (Vidal et al., 2007).

Further evidence for a role of mtROS in PCD comes from a recent finding that spontaneous PCD lesion formation in the *mod1* mutant with deficiency in fatty acid biosynthesis can be suppressed by genetic impairment of Complex I (Wu et al., 2015). Also, treatment with Complex I inhibitor rotenone could partially suppress PCD. This work indicates that deficiencies in Complex I can result in decreased ROS production and compromise HR and resistance against bacteria (Wu et al., 2015). To address whether mtROS or ATP status regulate PCD, the authors showed that overexpression of cytosolic Cu/Zn SOD could suppress PCD, suggesting that ROS is indeed causative (at least potentially mitochondrial ROS that reaches the cytosol). This study also indicated that the ROS generated by the plasma membrane NADPH oxidase is not directly involved in *mod1* PCD, emphasizing that formation of ROS through the mtETC likely initiates the PCD process (Wu et al., 2015).

Complex II subunit SDH1-1 was found to act as intermediate in a signal transduction pathway that induces promoter activity of salicylic acid (SA)-responsive plant defense genes (Gleason et al., 2011). Moreover, the effect of the mutation could be overcome by triggering promoter activity with exogenous H_2O_2, suggesting that the effect of SDH1-1 lies upstream of ROS presence. Indeed, this mutant showed less mtROS production and was more susceptible to several pathogens (Gleason et al., 2011), indicating the importance of mitochondrial function in pathogen defenses via ROS production. The *slo2* mutant, for which reductions in Complexes I, III, and IV have been reported along with increased ROS levels, is also more susceptible to the necrotrophic pathogen *Botrytis cinerea* (Zhu et al., 2014).

AOX has also been reported to be involved in plant defense against bacterial, viral, and fungal pathogens via regulation of ROS metabolism (Cheng et al., 2011; Lee et al., 2011; Cvetkovska and Vanlerberghe, 2012, 2013; Zhang et al., 2012). Differential expression of *AOX* and *MnSOD* transcripts at pathogen exposure and HR indicate a complex response pattern (Cvetkovska and Vanlerberghe, 2012, 2013). Silencing of AOX resulted in an increase of detectable matrix superoxide (Cvetkovska and Vanlerberghe, 2012). Furthermore, in AOX RNAi lines the threshold for cell death at inhibition of the cytochrome pathway by AA was lowered (Cvetkovska and Vanlerberghe, 2012, 2013), while AOX overexpression prevented HR and plant cell death (Vanlerberghe et al., 2002).

Non-mtETC proteins have reported roles in plant pathogen defense. A gene encoding mitochondrial inner membrane protein, *PAM16*, connected to the TIM23 translocase complex was identified as negative regulator of R-protein (Huang et al., 2013). The *atpam16* mutant has increased levels of *PR1* and *PR2* transcript, increased ROS production as measured by a luminol-based assay and 3,3′-diaminobezidine staining, smaller rosette size, and enhanced resistance to virulent pathogen attack (Huang et al., 2013). AtPAM16 may be involved in the import of a negative regulator of plant immunity to the mitochondrial matrix, which could function as a modulator of ROS generation (Huang et al., 2013). Plants overexpressing the mitochondrial outer membrane AAA ATPase *AtOM66* also have increased basal ROS production (NBT staining) and expression of SA marker gene *PR1* (Zhang et al., 2014a). AtOM66 overexpression plants show increased SA content and accelerated cell death rates and have altered pathogen resistance (Zhang et al., 2014a). The Arabidopsis defense protein PEN2 has recently been

found to hyper-accumulate on the outer membrane of mitochondria that cluster around infection sites of leaf epidermal cells (Fuchs et al., 2015). Redox sensing revealed an oxidative shift in the matrix glutathione pool of mitochondria at the infection site in relation to other parts of the same cell or neighboring cells. This observation is suggestive of specialized functions of mitochondrial subpopulations within a cell, which may include strictly localized ROS-linked immune responses.

In tomato, mitochondrial alpha-ketoglutarate dehydrogenase (Slα-kGDH) E2 binds SA, and *Slα-kGDH E2* silencing increased resistance to tobacco mosaic virus in an AOX-dependent way (Liao et al., 2015). Finally, a role of mitochondrial Pro metabolism in mtROS production and PCD has been emerging. Pro levels increase during HR, and the transcripts of *Pro dehydroge*nase (*ProDH1* and *ProDH2*) are induced by SA in Arabidopsis (Cecchini et al., 2011). Furthermore, silencing of *ProDH1* and *ProDH2* in Arabidopsis reduces ROS levels after pathogen infection but makes plants more susceptible to avirulent pathogens (Cecchini et al., 2011; Senthil-Kumar and Mysore, 2012). Similarly, silencing of mitochondrial Orn delta-aminotransferase and Pro dehydrogenases in *N. benthamiana* delayed occurrence of HR and favored nonhost pathogen growth (Senthil-Kumar and Mysore, 2012). How ROS is produced by Pro metabolism is not entirely clear, with some suggestion that pyrroline-5-carboxylate (P5C) to Pro cycling is indirectly responsible or that P5C itself can cause ROS and PCD (Qamar et al., 2015). For instance, overexpression of P5C dehydrogenase increases the resistance of plants to P5C-induced HR-like PCD and ROS production (Miller et al., 2009).

CONCLUSION AND PERSPECTIVES

Plant mtROS are primarily produced, and their rate of production is controlled, by the kinetics of the respiratory electron transport chain. However, mtROS biology still relies on an eclectic collection of indirect detection techniques, many of which have known shortcomings. A large literature based on mutant studies and transcriptional profiling argues that mtROS represent a hub in a highly interconnected network associated with abiotic stress, defense responses, hormone signaling, intracellular signal transduction, cell death, and development in plants. Significant progress has been made in identifying nonmitochondrial components involved in perpetuating putatively mtROS-dependent processes. However, the mitochondrion itself has remained a black box in terms of identifying a sensor or signaling component that can transmit mtROS signals into the cellular ROS network. Future studies will need to consolidate the mtROS literature by using the most appropriate ROS measurement method(s) to answer a specific biological question and provide additional forms of evidence to support the proposed role for mtROS in specific scenarios. These can include: measurement of concomitant alterations in indicators of oxidation such as the redox potential of mitochondrial thiols, physiological parameters associated with mitochondrial respiration such as matrix pH, metabolic products, or oxidative modifications of cellular biomolecules; genetic evidence based on targeting appropriate antioxidants or limiting ROS generation systems; and/or analysis of transcriptional responses that overlap with other reports or meta-analyses of oxidative challenge.

Key biological questions for the future include:

In which cell types does mtROS-mediated oxidation occur in vivo and what molecules exactly are oxidized by which reactive species?

How do biochemical oxidation mechanisms account for the specificity required for mtROS signaling to be involved in many distinct processes?

What is the physiological and evolutionary significance of the interactions between mtROS and plant hormone signaling?

Do mtROS have a specific role in PCD regulation and pathogen responses in plants?

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