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Oxidative signalling in seed germination and early seedling growth: an emerging role for ROS trafficking and inter-organelle communication

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Abstract

Underground early development of higher plants includes two distinct developmental processes, seed germination and then skotomorphogenesis, a mechanism which favours elongation of the hypocotyl and helps the seedling to find light. Interestingly, both processes, which are regulated by plant hormones, have been shown to depend on reactive oxygen species metabolism and to be related to mitochondrial retrograde signaling. Here we review the recent outcomes in this field of research and highlight the emerging role of ROS communication between organelles and cell compartments. We point out the role of mitochondria as an environmental and developmental sensor organelle that regulates ROS homeostasis and downstream events and we propose future directions of research that should help better understanding the roles of ROS in germination and seedling emergence.

ROS homeostasis regulates early steps of plant life

Germination is a key phenological step in higher plants life since it allows the transition from a dry mature and quiescent seed to a growing and metabolically active seedling after protrusion of the embryonic axis through the seed surrounding structures [1]. The developing seedling will ultimately give rise to a young plant after autotrophy acquisition, but it will first follow a specific morphogenic plan called skotomorphogenesis in which hypocotyl elongation is promoted, chlorophyll synthesis inhibited and an apical hook develops to protect the meristem during emergence from the soil [2],[3]. In agriculture, emergence is the visible outcome of completion of both germination and early seedling development steps that both differ in their adaptation ability to environmental issues. Knowing at which stage seedlings

fail to establish under penalizing conditions (e.g. water stress, temperature stress...) is critical but poorly addressed, even though it has been proposed that seeds/seedlings are often lost post-germination [4]. A deep comprehension of emergence thus requires gaining fundamental knowledge on each of these two early steps of the plant life cycle.

Germination is triggered by water uptake by the dry seed, but its success depends on a myriad of regulating factors that individually impact the accomplishment of a wide range of cellular events. In particular seed germination is strongly influenced by environmental signals such as temperature, light, water supply and oxygen and by endogenous signals such as seed dormancy, an inability of a viable seed to complete germination under apparently favorable environmental conditions [1,5]. The integration of environmental and endogenous signals by the seed provides spatiotemporal information which drives the decision to germinate, or not. This regulatory system ensures that the seedling will develop in environmental conditions which allow plant establishment and the perpetuation of the species through a new generation. The plasticity of this system requires that environmental (i.e. external stimuli) and developmental (i.e. dormancy) inputs are translated into cellular events allowing seed-to-seedling transition, which is mostly governed by cell expansion [6]. The antagonistic relationship between the plant hormones abscisic acid (ABA), promoting dormancy, and gibberellins (GAs), promoting germination, and the activation of proteins that modify cell wall properties are among the mechanisms that play a major role in radicle protrusion [5]. Reactive oxygen species (ROS) are also widely acknowledged as key regulators of the metabolic and molecular events that drive seed germination. Many studies have demonstrated that there was a strong relationship between their accumulation and the ability to germinate (see [7], for review) leading to the concept of oxidative window for germination [8]. According to this hypothesis, ROS homeostasis regulates the potential to germinate and can transduce both environmental signals and dormancy status. However if ROS levels are too low or too high they can impair seed germination [8] but also other steps of plant growth and development [9]. In particular non-permissive conditions for germination, e.g. environmental stress, would trigger over-production of ROS which can prevent germination, and the involvement of ROS in response to various stresses is well documented in plants [10,11]. In contrast dormancy alleviation and subsequent germination would be associated with a regulated ROS production leading to oxidative signalling [8]. Depending on their level within seed tissues, it has been demonstrated that ROS could cross-talk with hormone signaling pathways (eg. ABA and GAs) to positively regulate germination, modulate the cellular redox

status and the related downstream events or directly oxidize cellular component thereby triggering oxidative stress and delaying seed germination (see [7], for review). Interestingly ROS have also been shown to be involved in various other developmental processes throughout the plant life cycle such as root, shoot and flower development [9]. Using specific redox sensors to monitor changes in mitochondrial and cytoplasmic ROS levels in non-photosynthetic tissues (roots) of seedlings, a transient oxidative burst was shown to be triggered by mitochondrial dysfunction upon treatment with an inhibitor of mitochondrial translation that induces alteration of skotomorphogenesis [12]. Thus, ROS could also play a role during skotomorphogenesis and therefore in underground early development, since in this context seedlings grow in an hypoxic environment [13], often reported as being associated with ROS generation, in which the plant hormone ethylene also plays a role [14,15]. The impact of oxidative stress on developmental regulation was also reported in mutants affected in mitochondrial respiration as *rpoTmp* (defective in the T7-phage-type organellar RNA polymerase shared by plastids and mitochondria) and *atphB3* (defective in the mitochondrial membrane protein PROHIBITIN 3), presenting strong induction of oxidative stress markers and alteration of the skoto-morphogenic programme [16,17]. It has also been indicated that ROS produced by the respiratory burst oxidase homologues (Rbohs, i.e. NADPH oxidase) might have a role in root development in *Lepidium sativum* seedlings [18].

Although changes in seed ROS production are well described in various environmental conditions and physiological status, their mechanisms of production and action are far from being elucidated. These mechanisms have been reviewed recently [7] and in seeds and developing seedlings, which are deprived of functional chloroplasts, membrane bound NADPH oxidases, mitochondrial electron transfer chain (mtETC), peroxisomes or cell wall related enzymes are frequently cited for being responsible of ROS production [7]. However formal evidence about the relative roles of these production systems is lacking. In a very recent study Jurdak et al. [19] demonstrated that the mtETC, but not NADPH oxidases, was the main system involved in ROS production in response of seed to ethylene, a dormancy release hormone. Interestingly, Merendino et al. [17] have also highlighted the involvement of mitochondrial signals in the context of dysfunctional mtETC in *Arabidopsis* skotomorphogenesis. These studies, along with others [12],[20], suggest that a better attention has to be paid to the spatiotemporal regulation of ROS production, both at the subcellular level and the whole organ level in germinating seeds and developing seedlings. Indeed, in the context of seed germination and subsequent seedling development, and more widely in plant

developmental processes, the signalling specificity of ROS has been poorly considered, although there is increasing evidence showing that ROS signalling and sensing relies on complex and temporal interactions between subcellular compartments and cellular organelles [20,21]. This is also particularly relevant with regards to the crosstalk between ROS and plant hormones for regulating seed germination, knowing that the responses to ABA and GA have been shown to occur within distinct cell types within the embryo [22].

We claim that understanding the multifunctional roles of ROS in seed germination and seedling development requires studying the dynamics of ROS production at the subcellular level, integrating the possible crosstalk between organelles and cell compartments. Future studies will have to decipher how exogenous and endogenous cues can be transduced to modulate the activity of these systems. We propose here possible future directions of research that should help to understand how ROS homeostasis controls seed germination and early seedling development.

ROS trafficking along the road to germination and early growth

The role of ROS trafficking in developmental responses is rising in plants [23–25] and its understanding should allow explaining how ROS or related signalling molecules can induce specific transcriptional responses with regards to their production source. In the context of seed germination, efforts have to be put forward to elucidate the spatiotemporal regulation of ROS generation under permissive and non-permissive conditions of germination. For example, although Rboh are widely proposed as being important for seed germination (see [7]) their direct role as ROS producer during this process has not been clearly formerly demonstrated yet. Very recently, Yang et al [26] found that *RbohC* was expressed in the endosperm cap germinating lettuce seeds, where ROS production is likely to induce cell wall weakening, but not in the elongating embryo. In vivo $\cdot\text{OH}$ production in the apoplast of endosperm caps causes scission of polysaccharides and subsequently induces cell wall loosening [27]. Similarly, Luo et al. [28] proposed that RbohD was involved in ROS production during Arabidopsis seed germination under salt stress condition, but they did not provide any *in situ* evidence of ROS production in seeds. In contrast, Jurdak et al. [19] used cell imaging approaches to localize ROS production sites within seed tissues by visualizing fluorescence of the fluorogenic dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and of the redox sensitive biosensors Mit-roGFP, which express the reduction–oxidation-sensitive green fluorescent protein roGFP1 in mitochondria. These authors showed that mitochondria were the major intracellular source of ROS production in the embryos of germinating and non-

germinating dormant seeds. In seeds undergoing germination only, i.e. treated with ethylene in this study, ROS were detected in the nuclei at the mid time to radicle protrusion, as already observed by Leymarie et al. [29], which suggests that ROS accumulation in the nucleus might be a prerequisite for seed germination. Such features indicate that oxidative signalling during seed germination would be linked to a spatiotemporal control of ROS production and sensory mechanisms, involving a crosstalk between various cellular compartments to transduce the signal downstream [20,30]. We propose that in seeds this system is highly dynamic and strongly influenced by the progress towards germination. ROS production in the early steps of germination would result from mtETC [31,32] but the late phase of this process would be also associated with apoplastic ROS production, as previously stated [7]. The cellular sites of ROS production during early seedling growth before autotrophy are less clearly identified, but they could involve mtETC and Rboh. The cellular mechanisms involved in propagating ROS signal between organelles or compartments are far from being known. Among ROS, H_2O_2 is the more stable (half life of 1 ms) and is frequently mentioned as being involved in signalling between different subcellular compartments and organelles [20]. It has been proposed to leave mitochondria through permeability transition pores [33] for diffusing into the nucleus via nuclear pores [34]. It has also been proposed that extensions from organelles could associate them to the nucleus thereby facilitating ROS exchange between organelles [25,35]. Trafficking between organelles could also involve other signaling molecules, which would be more stable during their transit in the cytosol than H_2O_2 , including redox-sensitive signal transducers such as heat shock transcription factors, for example [25,36]. However none of these mechanisms has been formerly evidenced in either germinating seeds or growing seedlings.

Retrograde signaling: sending the message to the nucleus

The communication between mitochondria and nucleus demonstrated in seed germination and subsequent skotomorphogenesis suggests the occurrence of retrograde signalling in these developmental phases. Retrograde signals carry information provided by organelles, such as mitochondria, to the nucleus, leading to targeted transcriptional responses, a process often described as mitochondrial retrograde regulation (MRR) or signalling (MRS). In MRR, mitochondria are the source of specific signalling molecules, presumably ROS, that relay information to the nucleus to modify the expression of nuclear genes [37,38]. In both systems the molecular actors of MRR have been identified, they include the transcriptional regulation

of *AOX1*, a well-known marker of the MRR response in plants [37,37,39,40], of *ANAC013* in seeds and of *ANAC017* in seedlings, respectively, [19][17], which code for two endoplasmic reticulum-bound transcription factors that are translocated to the nucleus after their proteolytic cleavage [41,42]. The occurrence of MRR must also be considered on a wider perspective with regards to its potential relationship with hormone signalling [43]. Indeed, the works of Jurdak and other laboratories have clearly demonstrated that ethylene signaling, which promotes both dormancy alleviation and skotomorphogenesis reprogramming, is linked to MRR [19],[44,45]. Given the paramount importance of plant hormones in germination and emergence, paying attention to the involvement of MRR in these processes appears as critical. For example ABSCISIC ACID INSENSITIVE4 (*ABI4*) is one APETALA2 (*AP2*)-type transcription factor which is widely described as a key player in the ABA-mediated inhibition of seed germination [46]. *ABI4* plays a key role in the regulation of primary seed dormancy by binding to the CCAC and/or CACCG cis elements causing a repression of the expression of ABA inactivating genes such as *CYP707A1* and *CYP707A2* (decline in ABA degradation) and enhancing the expression of *GA2ox7* to promote GA catabolism [46]. *ABI4* has been shown to be involved in mitochondrial retrograde signalling where it represses the expression of the MRR marker *AOX1a* by targeting the CGTGAT elements in its promoter [47], thus establishing a link between ABA, MRR and germination [48].

Mitochondria as a sensor of exogenous and endogenous developmental signals

The findings related to ethylene-mediated activation of germination and skotomorphogenesis reprogramming support the hypothesis according to which mitochondria, through ROS production and MRR, might play a key role in germination and in early development of seedlings in response to environmental conditions, thus shedding a new light to the role of this organelle. Functional mitochondria play a crucial role in seed germination since it is an energy demanding process and during seed imbibition a progressive transition into metabolically and energetically active mature mitochondria occurs [49]. We suggest that mitochondria are the major ROS producing system during early seed imbibition, and that conditions permissive for germination stimulate mit-ROS generation and downstream events. This allows us proposing that mitochondria and the respiration process do not only serve to supply energy but would also be involved in the regulation of a developmental process such as germination and early seedling growth. Ma et al. [49] recently pointed out that mitochondria could be play a role in the sensing of environmental factors allowing

germination. They showed that a mitochondria-localized small heat shock protein (GhHSP24.7) induced ROS generation by mtETC, thereby activating seed germination in response to temperature. Nonogaki [50] also hypothesized that a loop of intracellular signalling by the nucleus, plastid, and mitochondrion could play a major role in seed dormancy regulation, but without experimental evidence. In the system involving mtROS and MRR, AOX is likely to play a fundamental role. AOX is an enzyme involved in the control of ROS and nitric oxide (NO) homeostasis by preventing over-reduction of the mtETC [51]. Importantly, the main AOX isoform in Arabidopsis, AOX1a, mediates ethylene responses to break seed dormancy [19] and the developmental response in respiration defective etiolated *rpoTmp* seedlings [17]. These concordant results reveal a new role for AOX in the regulation of germination and skotomorphogenesis, the two early key steps for plant establishment. *AOX1a* over-expression is often used as a marker of MRR in *Arabidopsis thaliana* [40,42] since this pathway is associated with an upregulation of the alternative oxidase gene. The activity of the AOX is positively regulated by ANAC013 and ANAC017 located in the endoplasmic reticulum. Past studies performed in pea (*Pisum sativum*) germinating seeds and etiolated seedlings revealed that during hypoxia, respiration performance is altered in mitochondria and NO was shown to control respiration rate to prevent mitochondrial anoxia [52,53]. In addition, NO was shown to reduce ROS levels and accelerate germination in chickpea [54]. In Arabidopsis plants under hypoxia the levels of AOX proteins are highly increased [55]. In these conditions, AOX produces NO feeding into the haemoglobin-NO cycle that ultimately increases ATP production and improves tolerance to hypoxic stress [55],[56]. Plants that over-express AOX survive better in low oxygen conditions and recover healthy in the reoxygenation step, indicating the role played by AOX in the low oxygen metabolism despite the low affinity for oxygen when compared to cytochrome oxidase [57]. Therefore, understanding the relationship between mitochondria, AOX and germination/early development control is of a specific importance when seeds germinate in hypoxic conditions which prevent both germination [58] and opening of the apical hook [13], thus interfering with plant establishment. In laboratory conditions where the mitochondrial respiration was genetically affected, i.e. in respiratory mutants as *rpoTmp* and *rug3* (disturbed in complex I biogenesis), the limitation of respiration induces the triple-like response by promoting the abundance and capacity of the AOX1a enzyme in developing seedlings. AOX1a enzyme is therefore not just a bio-marker for stress [2] that accumulates as a side effect, but it represents a key component of the reaction network that triggers subsequent cellular responses in response to respiratory deficiency. Interestingly, retrograde mitochondrial signalling pathways

have been shown to play a key role in the acclimation and adaptation to flooding in *Arabidopsis* plants [59,60] an environmental condition affecting O₂ diffusion from the air into the soil and therefore respiration capacity [61]. How precisely the AOX1a enzyme induces the reprogramming remains to be elucidated, but one can imagine that especially under oxygen limitation high capacities of AOX1a activity could reduce alternative substrates, generating products that may serve as signalling molecules, such as nitric oxide [42].

Future perspectives to understand ROS signaling in seeds and seedlings

Going deeper into the understanding of the dynamics of ROS generation and trafficking, both at the tissue and at the cellular level, will bring significant novel insights in the regulation of seed germination and seedling emergence. Figure 1 summarizes the possible roles of ROS trafficking and inter-organelle communication in these processes. Novel imaging tools, such as appropriate reporter genes [62] and *in vivo* redox sensors [63] should help addressing these points. Many questions have to be resolved, ranging from elucidating the exact role of mitochondria in transducing environmental and hormonal signals to deciphering the mechanisms involved in translating an oxidative message from organelles to the nucleus. One should also consider the role of other cell compartments and/or organelles, such as peroxisomes, in ROS generation and trafficking. The function of AOX in the whole process from germination to seedling growth, in particular in hypoxia, should be properly addressed. The mechanisms of ROS production by the nucleus, if any, are far from being known, and require attention. At last it will be of a particular interest to investigate how nuclear ROS can induce a specific transcriptomic signature that modulate seed and seedling developmental programs.

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Legend for Figure

Figure 1. Model of ROS trafficking and inter-organelle communication during seed germination and early seedling growth.

Environmental signals and endogenous signals, such as dormancy status, or hormones such as ethylene, are integrated by the imbibed seed or the growing seedling at the level of the mitochondria, thus generating controlled production of ROS through the mitochondrial electron transfer chain (mETC). The role of peroxisomes in these developmental processes cannot be excluded but is not documented yet. ROS release from mitochondria may induce ROS accumulation within the nucleus leading to a specific transcriptomic signature, which notably involves over-expression of *AOX1a*. Mechanisms of ROS propagation from mitochondria to the nucleus (i.e. retrograde signalling) are not known but they can rely on the activation of secondary messengers and/or release of the transcription factors ANAC013 (seeds) or ANAC017 (seedlings) from the endoplasmic reticulum (ER) and their translocation to the nucleus where they activate *AOX1a* expression. Overexpression of *AOX1a*, and thus the increased AOX capacity, would also play a role in seed germination and formation of the triple-like response. Abscisic acid (ABA), which inhibits seed germination, represses the expression abundance of the mitochondrial response signalling marker *AOX1a*. In anterograde signalling, the nucleus encodes unknown components that regulate gene expression within mitochondria.

