6. Plant seed: A relevant model to study aging processes

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Summary. Seeds are the major form of dispersal of plants in the environment. Seeds of many plant species are exceptionally adapted to harsh environmental conditions provided they are in a state of desiccation. Spectacular cases of seed longevity have been reported. It’s one of the singular case of pluricellular, differentiate eukaryotic organ able to survive several years in anhydrobiosis. Plant scientific community explore these fascinating aspects of seed aging thanks to the immense possibilities now offered to create/modify plants at a much faster rate and in a more accurate way than through classical and molecular genetic approaches and genomic tools. These investigations allowed unveiling seed specificities against aging processes.

Introduction

Aging in photosynthetic plants differs in some major ways from the process in animals. Plant biologists clearly differentiate aging from
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Senescence in higher plants is a type of programmed cell death included in a genetically determined developmental process [3]. The term “senescence” is consequently applicable to a process that leads to the death of a cell, an organ, or a whole plant and occurs at the final stage of its development [4]. Plant cells display either mitotic senescence or post-mitotic senescence [5]. Loss of cell division ability in the meristematic cells is called mitotic senescence or replicative senescence. On the other hand, post-mitotic senescence occurs in mature organs such as leaves, flowers and fruits. Unlike senescence, aging affects all living systems and can be defined in a population as an increase of the probability of death throughout time. It corresponds to the progressive alteration of normal biological functions and structural cell components resulting from a gradual accumulation of molecular damages that increases the susceptibility to environmental stresses. These changes will consequently have a direct impact on the functional integrity of organs, biological systems, and ultimately on the organism as a whole [6].

As in other kingdoms, survival of plant species mainly relies on the sexual reproduction, which gives birth to new individuals [7, 8]. Thus, the seed, resulting from this process, represents the main unit of dispersal and spread of flowering plants (Angiosperms). It contains an embryo (the future plant resulting from the fusion of the gametes), storage tissues (necessary for the establishment of the seedling) and a protective outer coat. According to their desiccation-tolerance properties, three main seed groups are differentiated: (i) viviparous-seeds, (ii) recalcitrant-seeds and (iii) orthodox-seeds. Several plant families of predominantly large-seeded tropical species are not tolerant to desiccation (viviparous-seed) or weakly tolerant to desiccation (recalcitrant-seed) and germinate prior to, or coincident with late seed maturation on the maternal plant [9]. Viviparous and recalcitrant plant conservation by seed storage is not possible. Indeed, these seeds rapidly lose viability if they are dried. On the other hand, in most plant species of temperate climates, so called “orthodox seeds” acquire tolerance to desiccation during their maturation on the mother plant and are dispersed in the soil in a quiescent dehydrated state [10]. This reduce metabolic activity allows seed survival in stressful environment sometimes during several years until the proper conditions for germination (transition from seed to seedling) will be encountered. If mature dry seeds are not collected for agronomical or industrial purposes, they will be stored buried in the soil after dispersal. The orthodox seed is consequently exposed to aging processes but is not concerned by senescence. As it gets older, the seed progressively loses its germination vigor and viability. In order to overcome adverse environments and optimize plant propagation “orthodox seeds” have consequently developed several resistance mechanisms responsible for an increased
longevity. Seed longevity can be defined as the time during which the seed will keep its aptitude to germinate. It mainly depends on the plant species, the genetic background, the storage environment and the quality of the seed lot. Spectacular cases of seed longevity have been reported. Indeed, radiocarbon dating allowed the determination of the age of date (*Phoenix dactylifera L.*) seeds at about 2000 years [11], sacred lotus (*Nelumbo nucifera*) seeds at 1300 years [12] or canna (*Canna compacta*) seeds at 600 years [13], all were found in the soil still alive and able to give progeny. These spectacular records of survival among eukaryotic living organisms make the seed an appropriate model to study the mechanisms allowing to cope with the progression of aging deteriorations. The comprehension of the survival mechanisms occurring during seed life and of theirs consequences in term of germination vigor is of paramount importance for seed industry and crop yield, as well as to manage seed conservation for the preservation of the plant genetic resources and biodiversity. Moreover seed physiology also provides experimental facilities to investigate aging. Seed aging can be artificially accelerated through a process called controlled deterioration treatment (CDT) and reverse by a pre-germination treatment that is called priming [14, 15]. Therefore, the physiological versatility of the seed has been extensively used as a powerful tool in functional genomics and genetic approaches that will be described, in order to document seed aging and their many controlling mechanisms of survival.

I. Seed life

Following maturation on the mother plant, the orthodox seeds are dispersed in the environment in a low hydrated state (5-10% moisture content) resulting from a developmentally regulated period of dehydration [16]. As a consequence of this desiccation, the cytoplasm condenses and intracellular components become more crowded leading to an increase probability of undesirable interactions [17]. It can indeed result in an alteration of the proteome or membrane fusion [17]. Following their maturation, seeds of many plant species can also display a period of dormancy when they fail to germinate under otherwise favorable conditions [18]. The exact requirements for dormancy breaking and subsequent germination depend on the species and thus contribute to the adequacy of the plant to its environment by delaying germination until the meeting of appropriate conditions for seedling development [19, 20]. As a result of this blockage, the time between the end of maturation and germination can be drastically increase and in the case of physiological dormancy so do the longevity.
One of the main problems from the seed point of view is that it can’t move by itself. Different evolutionary adaptations have enabled the optimization of seed dispersion through different means of transportation like wind or animals but finally most seeds will end buried in the soil. As a consequence, the seeds will have to cope with diverse biotic and abiotic stresses, proper to their direct environment, which can affect their viability and eventually participate in the progressive natural deteriorations induced by aging. But, it should be stressed that sudden loss of viability related to an instant damage as a mechanical stress, insect or animal attacks or sudden burst of temperature cannot be considered as aging alterations [21].

Among the numerous environmental factors known to affect seed longevity, the temperature and the moisture content appear to be the most important and this seems to be true both in controlled storage conditions and in the soil [22, 23]. Indeed, the longevity of a seed lot can be estimated quite accurately providing the temperature and the moisture content of the seed are known [24]. Moreover, it has been suggested that each 1% decrease in seed moisture content or 5-6°C reduction in temperature doubles the life span of the seed [25]. The rate of most biochemical reactions including theses implicated in the respiration process is strongly correlated to these two parameters. The lower the seed temperature and moisture content is, the slower the metabolism will be. The exact interplay between these two parameters is complicated and remains to be detailed. In the nature, the environment surrounding the seed can buffer variations in temperature and humidity related to the climate. The preservation of low moisture content is essential for seed survival in case of exposition to sub-zero temperature. Otherwise, uncontrolled freezing might cause fatal damage to the structure [26].

Different experiments carried in controlled conditions have shown that most of the extrinsic factors are of negligible importance in cold dry storage of orthodox seeds [21]. But, in the field, numerous constraints such as the pH of the soil, the salinity, the oxygen availability, the light, the presence of toxic compounds and microorganisms can promote seed aging [16]. Seed structure also has an important influence on the aging rate (ratio surface area/volume, hardness and permeability of the seed coat, presence of mucilage…). Moreover, the large heterogeneity and inequality of seeds to retain their germination vigor and viability among plant species and even among seeds within a lot highlight the importance of the genetic component in the determinism of seed longevity [27]. Seed deterioration is inexorable and the best that can be done is to control its rate [28].

Under aerobic conditions, a slow progressive alteration of cell constituents (lipids, sugar, proteins, nucleic acids…) has been reported [29].
Many of these damages have been associated to the deleterious effect of reactive oxygen species (ROS) due to their high reactivity towards biomolecules [28, 30-37]. Indeed, most of the modifications observed during aging including lipid peroxidation, nucleic acid alteration, enzyme inactivation and protein degradation can be the result of oxidative events induced by ROS [38, 39]. In plants, it has been described that developmental processes and/or environmental stresses can induce endogenous ROS production [40-42] though the mechanisms involved in their generation in orthodox seeds remain elusive [38]. Besides their metabolically quiescent state, dry seeds can endure several non-enzymatic oxidative reactions such as lipid peroxidation [28, 39, 43, 44], protein carbonylation [45, 46] or Amadori and Maillard reactions [47-51] leading to a progressive accumulation of ROS associated damages during storage. This results in a weakness that can be fatal to the embryo during imbibition.

If the seed encounters suitable conditions for germination during its life, it may, if still viable, fulfill its purpose and release the young seedling. But as a consequence of the aging process, the seed germination vigor can be severely affected. In other word, the capacity of a seed lot to germinate rapidly, uniformly and in a wide range of environmental conditions can be impaired or destroyed. As the seed germination process mainly relies on stored mRNA and proteins [52], damages at the DNA level can result in an aborted development of the seedling.

II. Morphology and physical structure of seeds

During its life a seed can be exposed to numerous adverse conditions reducing the embryo lifespan. To ensure the reproductive success of the species, plants have consequently developed mechanisms to enable long-term seed survival in soil. Some of these rely on the integuments and the properties of the glassy state of the cytoplasm.

The seeds of flowering plants consist of three genetically distinct components: (i) the embryo resulting from the fertilization of the egg cell in the embryo sac by one of the male pollen tube nuclei, (ii) the usually triploid endosperm (about 70% of angiosperm species) formed by the fusion of the two polar nuclei with the second spermatid nucleus, and (iii) the seed coat (or testa), representing the maternal tissues of the ovule, formed by the layers of the inner integument and the inner epidermis of the outer integument [53].

Due to its physico-chemical properties, the integument is of great importance for the preservation of the embryo for which it acts as a shield. Thanks to its hardness resulting from the strong drying and compression of the cell layers constituting the testa at the end of seed maturation, it represents a physical
and chemical barrier that enable the seed to withstand environmental attacks which could otherwise alter its structure [54, 55].

In most seeds, one of the very important features of the testa is its brown color conferred by the presence of phenolic compounds [56]. It results from the flavonoids oxidation occurring during the later stages of seed maturation. This high concentration of flavonoids has often been associated with resistance to fungi [57-62] due to their antifungal and antinutritiollnal properties [58, 63] allowing the seeds to maintain a healthy testa, byword for longevity. Other studies emphasize the importance of the impermeability of seed coat in particular to avoid solute leakage from the seed towards the medium, which has the effect of increasing the probability of pathogen infection [64], but also to regulate the absorption of water that can be deleterious to the embryo during imbibition [65]. For instance, in *Vicia faba*, tanin concentration has been shown to be correlated with the rate of water absorption [66]. Thus, in legumes, unpigmented seeds deteriorate faster and are more susceptible to imbibition damage upon germination [67, 68].

As discussed above, oxidative stress and reactive oxygen species (ROS) play an important role in seed aging processes during storage. It’s consequently interesting to note that the polyphenols of the seed coat also act as antioxidants and protect the embryo against oxidative stress [69, 70]. Moreover, flavonoids act as a UV screen to protect the inner tissues from ROS production by this mean [71-75]. Thus, the physico-chemical properties of the seed coat associated with the low moisture content (slowing down enzymatic activities) tends to limit the production of ROS in orthodox seeds.

The particular structure of the cytoplasm also participates in the protection against oxidative alterations. The seed completes its development, for most species growing in temperate climates, with a desiccation phase during which the cytoplasm switches from the liquid state to a glassy state [76-79]. The removal of water induces a super saturation of cytoplasmic components leading to an increase in cohesive forces between molecules and a decrease of molecular mobility in the cytoplasm [80, 81]. This glassy state corresponds to a relatively stable phase, near a solid except that it has properties of physical disorder of liquids. Even if the composition of the glass has been well studied there are still many uncertainties [79, 82-87]. It’s a complex structure in which different compounds interacts including sugars and proteins [82, 88, 89]. The main role of the glassy state in the protection of the seed embryo is the preservation of structural integrity of macromolecules [90-92]. To illustrate that, it has been demonstrated that the secondary structure of proteins in dry seeds of wheat is very stable and can be conserved during storage even after total loss of seed viability [93]. The
cytoplasm viscosity has also an impact on the diffusion of the ROS within the cell, resulting in an action range restricted to the targets closest to the production site [94]. A very strong correlation has been demonstrated between the intercellular molecular mobility, the moisture content and seed life span [80, 81, 87]. To conclude, the glassy state is very important to prevent seed deterioration [49, 84, 87].

### III. Chemical and biochemical feature of seed

Since orthodox seeds are dispersed in natural environment in a dehydrated state, waiting for suitable germination conditions, the occurrence of oxidative injuries is reduced. However, the low water content in the embryo is also limiting for certain biochemical activities that are required to limit age related cellular damages and enable seed survival during long-term storage. It has been hypothesized that enzymatic detoxification and repair mechanisms are decisive to limit molecular damage at the end of seed development and to repair and renew macromolecules and cellular structures until seed stored in soil encounter hydrated conditions that could induce germination and growth [30].

A large panel of antioxidant components accumulates in dry seeds during the late maturation step on the mother plant and contributes to control seed storability. As discussed previously, flavonoids localized in the testa play an important role in seed protection during storage. Flavonols are also abundant in the embryo where they can apply their protective action by scavenging ROS and protecting membranes. Recent papers reported that germination changed the distribution profile of isoflavone [95, 96]. For instance, in soybean seeds, the total flavonoid aglycones content increased by five times during germination, whereas acetyl-conjugated forms remained at a low and constant level [96]. Flavonoid aglycones should be hydrophobic enough to incorporate into cell membranes and lipoprotein lipids and interfere with lipid peroxidation [97]. It is very likely that flavonoid protective role at the cell level is not due exclusively to their antioxidant and prooxidant properties. Indeed, inductive or signaling effects may occur at lower concentrations than these required for effective radical scavenging. Further research will be needed to reveal in more detail the true signaling pathway of flavonoids in plants.

The $\alpha$-Tocopherol is the member of the vitamin E group ($\alpha$-, $\beta$-, $\gamma$-, and $\delta$-tocopherolsand tocotrienols) with the most biologically significant properties. Members of vitamin E group are associated with antioxidant function [98]. These lipophilic molecules are very abundant in seeds in particular in oleaginous seeds (Arabidopsis, sunflower, rape, olives, palm,
The primary function of vitamin E in plants is to limit non-enzymatic lipid oxidation during seed storage, germination and early seedling development. When \(\alpha\)-tocopherol is attacked by lipid peroxy radicals, it becomes, via one-electron oxidation, the \(\alpha\)-tocopheryl radical. The ascorbate–glutathione cycle recycles tocopheroxyl radicals to tocopherols. As a consequence of two-electron oxidation, \(\alpha\)-tocopheryl radical becomes \(\alpha\)-tocopherylquinone [99]. It was observed that the ratio \(\alpha\)-tocopherylquinone/\(\alpha\)-tocopherol increased in general with increasing oxidative stress. Also, recent works tend to demonstrate that tocopherols influence cellular signaling in seed and may modulate gene expression in the nucleus by affecting lipid peroxidation, and therefore the levels of oxylipins such as jasmonic acid [100, 101].

**IV. Genetic approaches to identify genes involved in seed survival: QTL**

The advent of molecular markers and QTL (Quantitative Trait Loci) mapping has opened a new insight for improving agronomic species especially for multigenic and quantitative traits such as yield [102, 103], resistance or tolerance to biotic and abiotic stresses [104, 105] or nutritional quality [106, 107]. A large number of studies have been done on Rice, which is one of the most important crops worldwide, as it feeds over half of the world's population. Rice seed vigor and longevity traits were strongly concerned. It has been described a rapid deterioration of this crop under humid tropical regions [108] and a poor germination of aged seeds under anaerobic conditions [109]. Therefore, in a direct seeding system, breeders have been working since the last decade on the selection of age-tolerant rice varieties to ensure consistent quality in fields. QTL analyses conducted in parallel on rice [110] and Arabidopsis [111, 112] showed that longevity is controlled by several genetic factors. The resistance to stress conditions during seed storage is one aspect of longevity. Therefore it would be coherent to observe a co-localization between QTL for longevity in Arabidopsis and QTL associated with environmental stress tolerance such as germination in saline conditions [112], in drought conditions [113] or under low temperature [114, 115]. The presence of genes implicated in ROS detoxification in these regions like catalase, superoxide dismutase, and a gene homolog to glutathione S-transferase is in accordance with the assumption that seed deterioration is mediated by the production of ROS. Interestingly, a co-localization between QTL of longevity and QTL controlling oligosaccharide contents (sucrose, raffinose and stachyose) has been pointed out [111]. This
result could be expected keeping in mind that these compounds are known to play a role in membrane and macromolecule protection upon desiccation and storage [116, 117] and also due to their ability to form glassy state in the cytoplasm [78]. Vigor and longevity are traits of a complex nature and it is therefore interesting to proceed by quantitative genetic methods but it is now necessary to continue this work with fine mapping and study of candidate genes by analysis of mutants to understand genetic mechanisms of these traits.

V. Application of high-throughput genomic analysis in order to identify candidate genes involved in seed longevity

High-throughput genomic analyses of expression profiles were realized by transcriptomic [118, 119], proteomic [45, 52, 120, 121] and metabolomic [122] to unveil complex molecular mechanisms involved in seed germination. About 12,000 mRNA species were quantitatively detected in Arabidopsis dry mature seed [118]. These transcripts correspond either to the residues of the maturation program or those that are necessary for starting of the germination program. It has been demonstrated that the germinating Arabidopsis seed has a distinctive transcriptome pattern, divergent from those found in other plant organs such as roots, leaves, hypocotyls, siliquae and flowers [123]. A proteomic investigation of the joint liability of transcription and translation in seed germination concluded that only protein translation is indispensable for radicle emergence suggesting that germination-specific proteins are translated from stored mRNAs [52]. However, if de novo mRNAs synthesis in not required for germination, transcription appear necessary to enhance seed vigor. In addition to storage proteins, three major group of gene products (mRNA and proteins), corresponding to metabolism, protein synthesis, folding and stability and stress responses are predominant in dry seeds and synthesized during early stage of seed germination. Interestingly, proteins associated with stress responses cover a large range of plant defense mechanisms such as detoxification (i.e.: reactive oxygen species, reactive nitrogen species, cyanide...), cell repair, chaperones or pathogenesis related proteins.

Seed storage proteins, localized in protein storage vacuoles (PSVs) constituting protein bodies, are degraded by hydrolytic enzymes to generate amino acids required for seedling growth. These enzymes are synthesized de novo in germinating seeds and transported to the PSV through the lytic vacuole (LV) pathway [124]. Previous studies demonstrated that vacuolar sorting receptor (VSR) proteins are associated with seed germination because
VSR knockout Arabidopsis seeds are not able to germinate [125, 126]. The role of these storage proteins is not limited as a source of nitrogen but also as major reactive radical scavengers that prevent cellular damages [45, 46, 121]. Recently, lower germination rates of Arabidopsis mutants upon application of oxidative and/or osmotic stress strongly suggested the involvement of specific genes in stress response [127-129]. A close relationship seems to exist between the abundance of characteristic proteins and seed longevity [46, 130].

VI. Biotechnology tools for candidate gene identification and validation

In order to confirm the involvement of candidate genes identified, several mutants have been analyzed. Control deterioration treatment (CDT) allows to artificially accelerate seed aging [14] and has been demonstrated to mimic molecular and biochemical events occurring during natural aging [46, 112]. Therefore, it constitutes a powerful tool to characterize the impact of mutations on seed longevity. However, up to date, due to the heaviness of a large-scale genetic screen based on CDT, no large mutant collection has been directly screen for defect in seed storability. Indeed, most of the seed longevity phenotypes have been characterized in already identified mutants mostly in the Arabidopsis plant model species or by reverse genetic approaches.

The reduce longevity of transparent testa (tt) and banyuls (ban) mutants [131] altered in the flavonoid synthesis pathway and consequently in the composition of the testa is in accordance with the protective role attributed to these compounds. It has also been shown that mutants with defects in the integument (ats, aberrant testa shape ; ap2, apetala2 ; gl2, glabra2) display an impaired viability phenotype during storage, underlining the importance of the tegument integrity [112, 131, 132].

Deficient mutants for antioxidant biosynthesis such as vitamin E (vte1 and vte2) also exhibit aging phenotype in CDT treatments [133]. The orthodox seed is well equipped to control the prooxidant/antioxidant balance in order to prevent damages of cellular content. Nevertheless seed sensitivity phenotypes to aging treatments for other mutants affected in free radical detoxification such as vitamin C deficient (vtc1-1) mutant, catalase double mutant (cat1cat3), or glutathione deficient mutant (cad2-1) are not clear [112]. Although these mutants still accumulate residual amount of the antioxidant or of the detoxifying enzyme targeted by the mutation, it is thought that specific ROS scavengers, such as vitamin E, peroxiredoxin or superoxide dismutase act predominantly in seed [134].
Recently, genetic modulation of the Protein L-isoAspartyl Methyltransferase 1 (PIMT1) using T-DNA insertion mutant and transgenic lines [135] allowed to demonstrate that protein repair of age related protein damage contributes importantly to seed longevity. PIMT enzyme activity reduces the level of deamidated and isomerized aspartyl and asparaginyl residues that represents a significant part of the spontaneous damage to proteins [136]. Alteration of protein conformation by isoaspartyl (isoAsp) accumulation has been described as a root case of seed aging. Although oxidative stress is known to enhance these protein alterations, isoAsp formation is not chemically an oxidation. This suggests that not only oxidative process contribute to aging. How this metabolism of protein modification control seed survival remains to be elucidated. The identification of altered proteins that are repair by PIMT will be promising to discover important actors of seed longevity.

Mutants affected in seed development such as leafy cotyledon1 and 2 (lec1, lec2) and fusca3 (fus3) or impaired in the plant hormone abscisic acid (ABA) synthesis and signaling such as aba1, abi3 and green seed (grs, enhancer of abi3-1) [14, 137-139] are also afflicted in seed longevity. It has been documented that abi3 and lec1 seeds are impaired in the accumulation of seed storage protein accumulation that could have protective property for seed cellular compounds. Nevertheless resistance mechanisms that are controlled by these loci remain to be understood. A recent screen for suppressor of abi3-5 (sua) aging sensitivity phenotype permitted to select 4 interesting mutants and their molecular characterization will be helpful to describe subtle controlling processes of seed storability [130]. Interestingly seed-specific overexpression of the heat stress transcription factor HSF in tobacco (Nicotiana tabacum) both enhances the accumulation of the heat shock protein HSP101 and small HSPs and improves resistance to controlled deterioration [140]. These findings point out the importance of the control of the expression of stress protein in seed longevity.

**Conclusion**

Plant seeds belong to the most impressive examples of organism longevity in eukaryotes and provide a suitable model to study resistance mechanisms against aging. This system used along with the diverse techniques available on plant material such as functional genomics, insertion mutant lines and quantitative trait locus (QTL), constitutes an extremely powerful tool to study aging. Therefore, the seed constitute a pertinent model to dissect much further the complex mechanism associated with the longevity.
References

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Seed aging