

# Genomics: A Hallmark to Monitor Molecular and Biochemical Processes Leading Toward a Better Perceptive of Seed Aging and *ex-situ* Conservation

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## Abbreviations:

AFLP, amplified fragment length polymorphism  
AGO, argonaute  
miRNA, micro RNA  
NGS, next generation sequencing  
PCD, programmed cell death  
PIMT, protein L-isoaspartyl methyltransferase  
PRC2, Polycomb repressive complex 2  
RAPD, random amplification of polymorphic DNA  
ROS, reactive oxygen species  
SSR, simple sequence repeat  
TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling  
qRT-PCR, quantitative reverse transcriptional polymerase chain reaction

## Abstract

For human food security, the preservation of 7.4 million *ex-situ* germplasm is a global priority. However, *ex situ*-conserved seeds are subject to aging, which reduces their viability and ultimately results in the loss of valuable genetic material over long periods. Recent progress in seed biology and genomics has revealed new opportunities to improve the long-term storage of *ex-situ* seed germplasm. This review summarizes the recent improvements in seed physiology and genomics, with the intention of developing genomic tools for evaluating seed aging. Several lines of seed biology research have shown promise in retrieving viability signal from various stages of seed germination. We conclude that seed aging is associated with mitochondrial alteration and programmed cell death, DNA and enzyme repair, anti-oxidative genes, telomere length, and epigenetic regulation. Clearly, opportunities exist for observing seed aging for developing genomic tools to increment the traditional germination test for effective conservation of *ex-situ* germplasm.

## Introduction

Seeds can be defined as embryonic plants that are enclosed in protective outer coverings, seed coats, along with stored food that provides nutrients for post-germination seedling growth. Seeds have both biological and economic importance, because they are required for reproduction in many plant species and almost all crop species that are used as staple foods (e.g., maize, wheat, rice, barley, and beans) are reproduced through seed. Thus, seeds are agents of plant dispersal and important contributors to human food security.

Over the centuries, many of the useful plants that have been domesticated are no longer used for commercial agricultural production and are becoming increasingly rare, as a result of many factors. This phenomenon is resulting in the loss of genetic diversity, which is a prerequisite for improving existing plant genetic resources. In

previous studies, Fu (2003), Fu and Somers (2009), and Nevo et al. (2012) used molecular biology and genomic techniques to demonstrate that both crop production and the food supply are vulnerable to genetic loss and that there is a need to increase the genetic diversity of various gene pools.

In many plant species, genetic diversity can be preserved for long periods through *ex-situ* conservation, in which seed is stored at a low temperature (-20 °C) and moisture content (Walters et al., 2005). However, not all seeds can be stored under these conditions, and based on their resistance to desiccation, seeds can be separated into two groups: (1) orthodox seeds, which can hold their germination potential over extensive intervals of dry storage, can sustain extreme desiccation by the end of their maturation, and are suitable for *ex-situ* conservation; and (2) recalcitrant seeds, which cannot survive the desiccation involved in *ex-situ* conservation (Roberts, 1973). Presently, ~7.4 million compliances *ex situ* are preserved in over 1750 gene banks worldwide (Dulloo et al., 2010), and~90% of these genetic resources are preserved as seeds.

During *ex-situ* conservation, the physiological activities of seeds are interrupted but not fully arrested, and the conditions under which the seeds are stored (e.g., low temperature and humidity) affect the ability of the seeds to germinate, although this also depends on their initial quality (Schoen et al., 1998; Walters et al., 2005; Kaviani, 2010; Arif et al., 2012; Huang and Song, 2013), and contribute to seed deterioration and aging over long periods.

Seed aging, which has been described as the loss of seed quality overtime (Coolbear, 1995), reduces seed vigor and viability and threatens the abilities of researchers and growers to maintain reliable genetic resources and to conduct effective crop production, respectively, especially for crops harvested during the vegetative stage (Tekrony and Egli, 1991; Ateinzar et al., 2006). In a previous study, the germination of more than 14 *ex situ*-conserved germplasm collections were tested, and low germination was observed for many of the collections, particularly for wheat, rye, buckwheat, soybean, and onion. For example, the germination of the wheat germplasm was reduced from 92 to 65% within 16 years of *ex-situ* conservation (Weber et al., 2005). These observations suggest that *ex situ*-conserved seeds are subject to reduced viability over time and that it is necessary to constantly

monitor seed aging in order to maintain viable seed stocks.

Currently, germination tests are used to evaluate the viability of *ex situ*-conserved seeds (Smith et al., 2003); however, the procedure is time-consuming and labor-intensive and fails to provide information about seed aging. Therefore, monitoring of seed aging needs speedy viability analysis, which should be innovative, low-cost, and equally reliable (Dona et al., 2013). Current progress in genomics and seed biology has unraveled new horizons for efficient handling and conservation of *ex-situ* seed germplasm under extensive storage period (Scheler et al., 2015). Therefore, genomic and biochemical markers could be used to monitor seed aging; however, comprehensive knowledge of the complicated network of molecular and biochemical processes that mitigate seed longevity is needed in order to select appropriate markers, which can then be used to gather useful information about the aging and viability of *ex-situ* seed stocks.

#### *Aim of this review*

Seed aging is a complex physiological trait that is controlled by a network of molecular and biochemical processes. At present, the basic strategy for monitoring seed aging is the accelerated, or artificial, aging test, which is also regularly used to investigate seed vigor. The molecular mechanism of seed viability and seed aging has largely remained unclear, until recently (Nonogaki et al., 2010). Therefore, traditional germination tests may be unable to diagnose the specific problems for seeds that are stored long term, and the development of some sensitive tools for monitoring seed aging has become critically important.

In recent years, our understanding of the molecular and biochemical processes involved in seed aging has progressed extensively, and discoveries regarding aging mechanisms, programmed cell death (PCD), and the role of mitochondria and epigenetics have improved our understanding of seed viability and deterioration. It has been identified by numerous inclusive reviews: reactive oxygen species, lipid peroxidation mediated by free radicals, imbalances in growth-regulating enzymes, enzyme activation or protein degradation, post-translational modification of proteins, impairment of metabolic transition, damage to genetic integrity, and commotion of cellular membranes as main factors of seed aging (Priestly, 1986; Smith and

Berjak, 1995; Walters, 1998; McDonald, 1999; Ventura et al., 2012; Rajjou et al., 2012; Table 1).

During the past two decades, extensive efforts have been made to understand the physiology of seed aging, but the genetic mechanisms underlying seed deterioration are yet to be identified (Nguyen et al., 2012). The aim of this review is to identify sensitive signals for seed aging from current development in seed cellular and molecular biology and genomics. Consequently, we can develop new genomic approaches for monitoring seed aging. We hope that our endeavor may bridge the gap between basic academic research and applied seed biotechnology so that *ex-situ* conservation can be used more effectively.

### **Seed desiccation tolerance and suitability for *ex-situ* conservation**

Seeds, which develop from double-fertilized ovules, represent the main reproductive strategy of flowering plants (Rajjou et al., 2012). Following double fertilization, both the embryo and storage tissue begin to grow and continue to grow until a

specific time, which depends on the particular plant species. When the development of the embryo is repressed on the mother plant and storage products are collected, by the aid of compound regulatory complexes that incorporate genetic programs, metabolic signals and pathways for hormonal signaling, seed maturation starts to acquire tolerance against desiccation (Ventura et al., 2012). However, not all seeds can withstand desiccation, and only orthodox seeds can be stored *ex situ* in their dehydrated state for long periods, which is regulated by complex networks of molecular and biochemical processes.

As opposed to recalcitrant seeds, the orthodox seeds activate various protective processes during maturation, and these processes can preserve DNA and the integrity of cellular membranes. These processes also facilitate the maintenance of desiccation tolerance (Smith et al., 1995; Berjak and Parmenter, 1997; Parmenter and Berjak 1999; Boubriak et al., 2000; Buitink et al., 2002; Weitbrecht et al., 2011). When 90% of cellular water in a seed is removed and the seed maintains the

**Table 1.** Disruptive changes and their consequences on seed aging and deterioration.

Disruptions	Associated Changes	Effects	References
Membrane degradation	Loss in membrane permeability and more electrolyte leakage	Decline in germination, vigour, as well as seedling emergence. Diminishing of cell function and energy production	Walters et al., 2010; Guan et al., 2014
Enzymes Alterations	Reduction in the activities of ribonuclease, lipase, protease, acid phosphatase, diastase peroxidase, amylase, DNAase, catalase and dehydrogenase enzymes.	Increasing production of ROS and H <sub>2</sub> O <sub>2</sub> and seeds become more receptive for the oxidation of PUFA present in membrane.	Biabani et al., 2011; Hancock et al., 2015; Jin and Pie, 2015
Changes in Cell Chemical Constituents	Decrease in total sugars, protein.	Decrease in oligosaccharides associated with membrane stability.	Verma et al. (2003); Hussain et al., 2015
Reduced Metabolic Activity	Reduction in metabolic activities	Fall in the ability to form nucleic acid and nucleotides	Astegar et al., 2011; Kumar et al., 2015
Free Radical Damage	ROS oxidizes fatty acids in the absence of enzymes.	Damage the components of cell which leads to deterioration of oil seeds	Shelar et al., 2008; Li and He, 2015
Chromosome Aberrations	Damage in chromosomal structure leads to expression alterations.	Promotion of seed ageing	Ghassemi-Golezani et al., 2010; Walters et al., 2010; Astegar et al., 2011
Collapse of functional structure	Fall in ATPs level, decrease in sugar content, failure of ribosome to dissociate, enzymatic degradation, and starvation of meristematic cells.	Increase in seed leachates and free fatty acids contents that lead to seed aging deterioration.	Ghassemi-Golezani et al., 2010; Santhy et al., 2014

capability to rehydrate effectively, it is considered desiccation tolerant. The molecular hydration shell is destroyed by reducing the moisture content of a seed to less than 23% (Smith et al., 2003; Ventura et al., 2012; Sreenivasulu et al., 2013); however, numerous cellular processes subsidize to facilitate desiccation resistant seeds in the dehydrated state, e.g., presence of antioxidant systems; accretion of protective biomolecules, such as LEA (Late Embryogenesis Abundant) proteins, disaccharides (sucrose), and other oligosaccharides; activation of repair mechanisms during rehydration; and increase of amphipathic molecules (Osborne, 2000).

In contrast, the impairment of metabolic pathways and cellular membranes occurs as a result of cellular water loss during the dehydration of seeds that cannot endure desiccation. In the dehydrated state, the rates of membrane damage, protein denaturation, loss of enzyme functions, and nucleic acid integrity are influenced by the genetic makeup of the seeds, as well as the surrounding environment. Thus, seed moisture content is a core factor that modulates the balance of cellular functions and integrity (Osborne, 2000). In an experiment with *Eugenia pleurantha*, germination was significantly reduced when seeds were gradually dehydrated to 30, 20, and 10% moisture content, and germination was completely inhibited when the moisture content of the seeds was reduced to 7%, with DNA seemed greatly degraded. However, in orthodox seeds, genetic stability was maintained at 7% seed moisture content. These observations support the hypothesis that, in orthodox seeds, the maintenance of DNA integrity during dehydration and DNA repair during rehydration are basic requirements for desiccation tolerance (Massetto et al., 2008). The DNA exhibits a specific conformation, but DNA enters different conformational states with the change in water content, and upon manifestation of dehydration, there is an increase in the number of base pairs per turn of the DNA helix. In response to water loss, the shift of DNA molecules to a more opposite conformation that can withstand dehydration is governed by various processes.

If chromatin or DNA conformation is not organized to facilitate dehydration, cellular damage can occur at the onset of dehydration. One important regulatory mechanism is the posttranslational modifications of histones, which ensures the appropriate chromatin structure (Ventura et al., 2012). In *Arabidopsis thaliana*, the HUB1 (Histone

Monoubiquitination) and HUB2 genes encode E3 ligases, which are necessary for the monoubiquitination of histone H2B. It has been hypothesized that chromatin remodeling via monoubiquitination plays a basic role in the maintenance or induction of dormancy by regulating abscisic acid concentration and sensitivity in seeds (Liu et al., 2007). Another possible mechanism of chromatin remodeling is protein glycation, which is a non-enzymatic reaction between amino groups of proteins and reducing sugars. The transition of DNA is facilitated by histone glycation to a conformation well-suited with the desiccated state (Talasz et al., 2002).

When seeds are stored *ex situ* for long periods, the mechanisms that induce desiccation tolerance weaken with the passage of time. This reduced desiccation tolerance results in seed deterioration, which then results in seed aging and reduced viability. Viable seeds should imbibe on exposure to water and proceed to germination. A considerable outflow of cellular solutes occurs when water uptake begins, and this accelerates germination, by reducing the concentration of inhibitors, and is thought to damage cellular membranes, as a result of rapid or heterogeneous rehydration. Seed testa play an important role in regulating water intake rate (Koizumi et al., 2008). In late stages of germination and during imbibition, water is transported and distributed within the seed tissue by membrane proteins that facilitate the passage of small non-polar molecules and water or aquaporins (Vijay et al., 2009).

In *Arabidopsis*, along with the upregulation of genes involved in transporting nucleotides to mitochondria by seed imbibition, a temporary upregulation of genes controlling mitochondrial DNA replication and translation is also observed. At the time of maturation, both membranes and mitochondrial enzymes are protected by LEA proteins (Tolleter et al., 2010; Law et al., 2012). Thus, a variety of cellular, molecular, and physiological processes contribute to seeds' ability to withstand desiccation and to resume germination under favorable conditions.

### **Recent developments in the science of seed aging**

Owing to their importance in plant reproduction, extensive efforts have been made to understand the molecular, biochemical, physiological, and metabolic processes involved in controlling seed development and germination, and many published reviews

describe the progress in the field of research (for detail see Bove et al., 2001; Chaudhury et al., 2001; Weber et al., 2005; Le et al., 2007; Linkies et al., 2010; Nonogaki et al., 2010; Rajjou et al., 2012; Ventura et al., 2012; Diaz-Vivancos et al., 2013; Sreenivasulu and Wobus, 2013).

Along with seed viability, seed vigor is another important parameter of seed quality. Seed vigor determines the strength and uniformity of the resulting seedlings. Recently, some PCR-based molecular markers, including RAPD, AFLP, and simple sequence repeat (SSR) marker shave been developed and are considered to be sensitive measures of seed vigor (Chwedorzewska et al., 2002; Vijay et al., 2009; Dona et al., 2013). However, in contrast to the rapid progress in understanding the molecular pathways and regulatory mechanisms involved in seed development, germination, and viability, the development of new technology for monitoring seed aging has progressed more slowly.

As a result, recent efforts have been increased, and the current understanding of the molecular and cellular processes related to seed aging has improved. For instance, the roles of reactive oxygen species (ROS) and anti-oxidative enzymes, PCD triggered by mitochondria and DNA alternation, molecular repairs (including DNA and protein), and epigenetic regulation are now beginning to be understood. Recently, it was reported that seed aging is related to cellular-level modifications, such as the loss of membrane integrity, RNA impairment, decreased energy metabolism and protein synthesis, and DNA degradation (McDonald, 1999; Corbineau et al., 2002; Kibinza et al., 2006; Ding et al., 2016); Osborne and Cheah (1978) and Osborne et al. (1981) reported the degradation of embryonic nuclear DNA during seed aging. However, DNA laddering commonly observed in active and genetically controlled PCD or unaddressed deprivation after intensive DNA oxidation may lead to DNA impairment (Stein and Hansen, 1999; Slupphaug et al., 2003).

### **Cellular and physiological processes involved in seed aging**

#### *Mitochondrial alteration*

Germination is a complex and energy-demanding process that requires the proper operation of mitochondria, which must survive desiccation over long storage periods and become rapidly functional after imbibition to meet the ATP demand of various

metabolic processes in germinating seeds. With the help of recent developments in the fields of molecular and cellular biology and molecular physiology, it is possible to validate the performance of mitochondria in aged seeds, up to a certain degree. During prolonged storage, mitochondrial dysfunction is supposed to be the main cause of seed aging. Meanwhile, in animal cells, mitochondrial alterations probably result in cell death (Bras et al., 2005) and are considered the main cause of agingin mammalian cells (Kujoth et al., 2005).

In the seeds of cereal crops, the biogenesis of mitochondria during germination is thought to proceed from the structural progression of already-present mitochondria, whereas in lipid storing seeds, mitochondria are generated through *de novo* synthesis (Morohashi, 1986; Priestley, 1986; Shatters et al., 1995). Logan et al. (2001) proposed that, during imbibition, mitochondria from dry maize seeds depend on the oxidation of cytosolic NADH to differentiate into functional mitochondria. Meanwhile, the relationship between mitochondrial performance and germination was determined in pea (*Pisum sativum* L.) by analyzing the physiological properties of mitochondria isolated from normal, primed, and aged seeds (Benamar et al., 2003). The authors found that priming increased mitochondrial performance, whereas accelerated aging deteriorated pea seeds by strongly affecting the oxidative properties of mitochondria, thus impairing their ability to synthesize ATP. The results also highlighted that the integrity of the outer and inner mitochondrial membranes was the primary target for aged-induced damage and recovery.

Moreover, the monitoring of mitochondrial membrane stability during complete germination period reveals that the recovery and integrity of the inner mitochondrial membrane is more sensitive to desiccation than that of the outer mitochondrial membrane (Wang et al., 2012). The permeability of the outer mitochondrial membrane triggers caspase-dependent apoptosis. However, the loss of its integrity results in the discharge of soluble proteins, like cytochrome C, from intermembrane space (Lo et al., 2011). Till note, no staunch evidence is available to authenticate the role of mechanical factors in the deterioration of outer mitochondrial membrane integrity, and apoptotic mechanisms vary from organism to organism.

Overall, we can conclude that the segregation of mitochondrial DNA and division of mitochondria is

facilitated by mitochondrial actin and that apoptosis or plant cell death is initiated by the dynamics of mitochondrial actin, which initiates the release of cytochrome C (Lo et al., 2011). These observations reflect the close association of mitochondrial membrane integrity and energy metabolism during aging. Thus, energy metabolism and seed deterioration are likely related, since the availability of energy determines seed viability and vigor. On the other hand, fluctuating moisture constituents have been reported to accelerate the impact of aging on the energy metabolism of artificially aged sunflower seeds (El-Maarouf-Bouteau et al., 2011), and the adenylate pool (ATP, ADP, and AMP) of these seeds was found to decrease markedly at higher levels of seed moisture level, as a result of mitochondrial dysfunction.

Mitochondrial alteration during early seed aging or germination is related to the recovery or damage of the inner and outer mitochondrial membranes. Recently, Wang et al. (2012) devised a strategy to quantify the recovery rate of the inner mitochondrial membrane after the imbibition of desiccation-stressed pea seeds; they used an oxygen electrode system to calculate the mitochondrial consumption of oxygen and found that the recovery of mitochondrial function and structure followed a cumulative normal distribution. Along with the physiological changes mentioned earlier, on imbibition of dry seed many molecular changes like increased expression of factors and genes regulating RNA processing and protein respectively, were also observed within mitochondria. Therefore, it can be deduced that these events are followed by an increase in the expression of genes that encode proteins responsible for the replication and translation of mitochondrial DNA. Moreover, the upregulation of genes for nucleotide synthesis in cytosol and nucleotide transportation into mitochondria has also been reported, and transcriptome analysis reveals a rise in transcript levels of genes that govern different metabolic and bio-energetic processes of mitochondria (Cao et al., 2015). Mass spectrometry and Western blot analysis of protein levels indicate a positive correlation between the proteome and transcription factors (Law et al., 2012).

#### *Programmed cell death (PCD)*

Lockshin and Zakeri (2004) described PCD as the sequence of (potentially interruptible) events that is associated with controlled cell death, and PCD can be generally described as apoptotic (type I) or autophagic (type II) cell death, in contrast to necrotic

(type III) cell death, which is a non-physiological phenomenon induced by injury or infection. At the cellular level, PCD has been accepted as a primary process and is involved in development, defense, and stress responses (Reape and McCabe, 2008). Initially, the observation of cereal seed germination and aleurone autolysis was used to elucidate the role of PCD in seed viability. Gibberellic acid and abscisic acid are strongly correlated with the regulation of seed viability and inhibition of PCD, respectively (Fath et al., 2000; Chmielowska et al., 2015). Moreover, it is also assumed that ROS initiate the fundamental processes of PCD; as ROS threshold levels are attained, the PCD signal transduction pathway is triggered (Kranner et al., 2010; El-Maarouf-Bouteau et al., 2015). However, the mechanism by which ROS-initiated PCD contributes to reduced seed viability and vigor during aging is mostly unknown (Hossain et al., 2015).

El-Maarouf-Bouteau et al. (2011) reported that PCD resulted in a 50% reduction in the viability of sunflower seeds that were subjected to accelerated aging under different relative humidity and temperature levels. Both RAPD and AFLP analyses indicate that genomic DNA deterioration is associated with seed aging (Chwedorzewska et al., 2002; Kranner et al. 2006; Vijay et al., 2009); RAPD is also a reliable technique for diagnosing DNA deterioration (Liu et al., 2005) and mutational events, including point mutation, rearrangement, deletion, insertion, and structural distortion in bacteria, animals, and plants (Atienzar and Jha, 2006; Vijay et al., 2009).

To further confirm the role of PCD in seed aging, El-Maarouf-Bouteau et al. (2011) used the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay, coupled with propidium iodide (PI; marker for cell death) and 4,6-diamidino-2-phenylindole (marker for living cells) staining. *In situ* observations (TUNEL and PI staining) can be used to study PCD within individuals, whereas DNA breakage and mitochondrial failure function at the population level. The TUNEL assay detects apoptotic cells, whereas PI staining reports the co-localized nuclei of dead cells. The bright green fluorescent spots, which% moisture content for 7 days at 35°C were subjected to PCD. Till note, no TUNEL reaction is evidenced in normal seeds. However, PI staining of seeds undergoing aging under the same conditions has confirmed reduced cell viability in the embryonic axis; El-Maarouf-Bouteau et al., 2011 reported that

a positive TUNEL assay indicated that the embryonic axes of sunflower seeds aged at 37°C, and TUNEL *in situ* hybridization maybe the most effective strategy for identifying PCD associated with seed aging. However, this strategy is relatively complicated and includes many steps, including material fixation, paraffin embedding and sectioning, TUNEL *in situ* hybridization, and microscopic examination.

In woody plants like elm (*Ulmus pumila L.*) seeds, PCD initiates the process of aging (Hu et al., 2012), and when these seeds are subject to controlled deterioration treatment, the electrophoresis of DNA extracted from the seeds exhibits typical DNA laddering, which is recognized as an important characteristic of PCD and apoptotic cell death in plant and animal cells, respectively. Western blot analysis has indicated that mitochondria begin releasing cytochrome C into the cytosol during the second day of controlled deterioration treatment. However, cytochrome C is absent in the cytosol by the fifth day. In addition, the activity of DEVDase and caspase-3 are significantly increased during controlled deterioration treatment. Thus, the whole cellular area of seeds follows PCD unanimously under aging conditions. Since the simultaneous occurrence of PCD causes seed death, this process cannot be labeled as a selective removal of dead cells, as reported for other developmental processes or in response to pathogens (Hengartner, 2000), and mitochondria are also observed during PCD alterations, which contributes to their malfunction, thus facilitating the occurrence of PCD. In plant and mammalian cells, the interruption of energy metabolism does not affect seed viability, which verifies that mitochondrial death occurs in the early phase of PCD (Atlante et al., 1996; Vacca et al., 2004).

#### *Reactive oxygen species (ROS) and signaling*

Chemically reactive oxygen species are widely considered key factors of seed aging under prolonged storage and are generated by the reduction of molecular oxygen ( $O_2$ ) to hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $OH$ ), singlet oxygen ( $^1O_2$ ), and superoxide ( $O_2^-$ ). Oxidative processes and the formation of free radicals are induced during various metabolic processes or under different stresses, such as dehydration. During the storage of dry seed, non-enzymatic mechanisms, such as lipid peroxidation, are thought to be responsible for the accumulation of ROS, owing to the absence of free water, whereas enzymatic mechanisms are responsible for ROS

production during imbibition. However, in seeds with dynamic metabolic activities, the mitochondrial respiratory chain is thought to be the main source of ROS generation (Bailly, 2004), and the deposition of ROS can also be accelerated by alterations in mitochondria (Wei and Lee, 2002; Cash et al., 2007; Li et al., 2015).

Previously, ROS production and lipid peroxidation have been reported to increase, along with moisture content, in response to artificial aging (Kibinza et al., 2006). Seeds possess higher concentrations of polyunsaturated fatty acids than other plant tissues (Mansfield and Briarty, 1992). Therefore, large amounts of ROS are formed and deposited as a result of the peroxidation of polyunsaturated fatty acids (Bailly, 2004). Reactive oxygen species contribute to seed aging by triggering protein oxidation, membrane perturbation, mitochondrial dysfunction, and genetic damage (Coolbear, 1995; Khan et al., 2015) and are believed to induce porosity in mitochondrial membranes, which then initiates apoptosis, owing to the discharge of cytochrome C and other apoptogenic components into the cytoplasm (Tatton and Olanow, 1999). The intensity of cellular damage caused by ROS depends on the ability of seeds to eliminate oxidative agents via enzymatic and non-enzymatic antioxidant systems. In fresh seeds, harvested from various rice genotypes, the total antioxidant potential is under direct genetic control; however, in aged seeds, environmental factors might play a role, as well (Talai and Sen-Mandi, 2010). The enzymatic anti-oxidative system comprises a number of enzymes, including ascorbate, catalase, superoxide dismutase, and glutathione reductase. These major antioxidant enzymes can scavenge excessive ROS from within plant tissues (Bailly, 2004) and use different mechanisms to remove ROS from the organelles or cells. During the germination of several plant species, superoxide dismutase activity plays a critical role in balancing ROS at non-toxic levels (Wojtyla et al., 2006). In tobacco (*Nicotiana tabacum*), overexpression of the superoxide dismutase gene alleviates seed deterioration during aging (Lee et al., 2010), and the expression of various anti-oxidative genes in pre- and post-germinative pea seeds has been elucidated in response to seed aging (Yao et al., 2012). Moreover, genes located in the cotyledon and embryo axes of germinating seeds are highly expressed, and in young seedlings, expression was detected in cotyledons, roots, and shoots. On the other hand, the rapid aging of pea seeds reduces seed viability and seedling growth. Deviation in transcriptional

activation of eminent antioxidant genes indicates a positive correlation with this effect (Yao et al., 2012). Accelerated lipid peroxidation from oxidative stress reduces the activities of anti-oxidation enzymes that play important roles in seed aging.

In sunflower seeds, reductions in seed viability and vigor that occur during aging are associated with accumulation of hydrogen peroxide, accelerated lipid peroxidation, and reduced activity (Kibinza et al., 2006; Nagel et al., 2009; Li et al., 2015). Metallothioneins (MTs) serve as signaling molecules and ROS scavengers, both within and outside the nucleus, (Wang et al., 2010), which highlights their potential interaction with DNA repair machinery (Balestrazzi et al., 2011). Since signals monitoring seed aging can be classified into five categories: molecular, biochemical, physiological, metabolic, and mitochondrial (Fu et al., 2015), we have devised an integrated model that elucidates the pathways exhibiting positive and negative effects of these signals on seed aging and germination (Figure 1). These signals are highly complicated and interconnected; therefore, it is difficult to tag specific fingerprints of various aging stages. Balestrazzi et al. (2009) provided convincing evidence of the putative protective roles of MTs in the nucleus. The researchers demonstrated that the *Pisum sativa PsMTA1* gene encodes an MT-like protein that eliminates the risk of oxidative DNA damage by conferring protection against oxidative stress. In *Arabidopsis thaliana*, seed-specific type-4 MT (MT4a and MT4b) mRNAs accumulates during late embryogenesis and rapidly vanish during imbibition, which highlights the possible role of MTs in desiccation tolerance (Kranner and Colville, 2010; Najdekorova et al., 2012). The carbonylation of proteins occurs in aged seeds following oxidative stress (Rajjou et al., 2008), which makes them susceptible to degradation, resulting in loss of activity. During imbibition, seed aging also induces the inhibition of the *de novo* synthesis of proteins, such as fructose-1,6-bisphosphate aldolase (FBPA), which is related to metabolic processes (Rajjou et al., 2008). In dry seed, water uptake is required to activate antioxidant enzymes and for germination to take place. However, this uptake reactivates various metabolic processes, thus contributing to the production and deposition of ROS, which may inhibit germination (Wituszynska et al., 2015). On the other hand, the production of ROS, such as H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, and hydroxyl (OH) radicals, is directly associated with germination (Schopfer et al., 2001). Therefore, successful germination strongly depends on effective anti-oxidative processes that can maintain

sufficiently low limits of ROS (De Gara et al., 1997; McDonald et al., 1999).

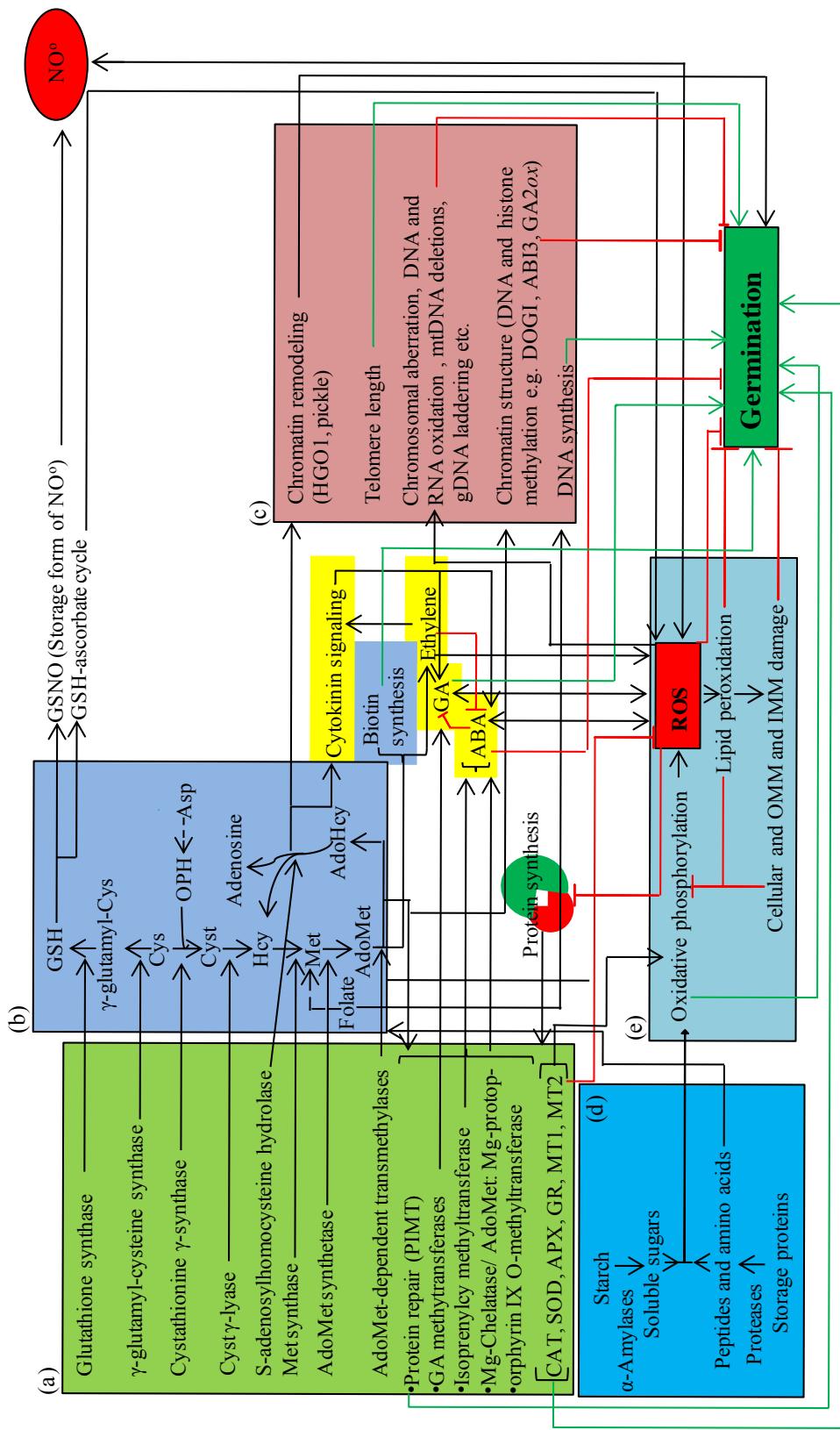
Although ROS are deleterious to cells and tissues, they regulate plant growth and development by loosening cell walls during cell elongation (Miller et al., 2008). In isolated segments of maize modulated ROS yields H<sub>2</sub>O<sub>2</sub>, which boosts leaf expansion (Rodriguez et al., 2002). Moreover, in germinating pea seeds high amount of O<sub>2</sub><sup>-</sup> deposits at radicle expansion stage (Kranner et al., 2010), which validates the role of O<sub>2</sub><sup>-</sup> in stimulating cell elongation (Passardi et al., 2004; Barba-Espin et al., 2011). Apart from their deleterious effects, ROS play a pivotal role in the signal transduction system of seed. Therefore, seed germination probably only occurs when the level of ROS is sustained under a critical threshold (i.e., oxidative window for germination), which then triggers ROS-mediated signaling pathways (Figure 1). Recently, Diaz-Vivancos et al. (2013) summarized new discoveries involving ROS and their interaction with growth regulating hormones in the regulation of seed germination.

### Molecular processes involved in seed viability

There are quite a few pathways that have been identified to play roles in seed germination, thanks to recent developments in genomics and molecular physiology (Rajjou et al., 2012), and some of them have been confirmed to be pivotal factors of seed viability.

#### DNA repair

Most DNA damage related to seed aging is a consequence of oxidative stress that occurs during desiccation and extensive periods of storage (Linkies et al., 2010). DNA damage results in single and double strand breaks, which subsequently results in reduced seed viability and seedling vigor; thus, DNA damage must be repaired during germination (Liu et al., 2005). Till now, little is known about the complicated repair mechanisms of DNA in plants. In plants, both nucleotide and base excision repair pathways are supposed to be the most prominent DNA repair processes. Various DNA ligases are involved in DNA repair. In the genomes of the nucleus, chloroplast, and mitochondria, replication, recombination, and repair processes are facilitated by multiple ligase genes. Investigation of the *Arabidopsis* genome has verified the occurrence of three DNA ligases, including two homologs (AtLIG and AtLIG4) of yeast/animal DNA ligases I and IV, respectively. However, a third ligase (AtLIG6) encodes protein domain specific to plant



**Figure 1.** Schematic presentation of interconnection and correlation between various biological signals during seed germination. In each of the signal category-specific examples, their effects on other biological signals and germination are presented. (→) Positive effects of particular biological signals on germination. (—) Inhibitory effects of particular biological signals on germination.

Abbreviations: glutathione (GSH); S-Nitrosoglutathione (GSNO); high osmolarity glycerol response 1 (HOG1); ABA insensitive3 (ABI3); GA3-oxidase (GA3ox); protein L-isopropiyl-O-methyltransferase (PIMT); gibberellic acid (ABA); reactive oxygen species (ROS); inner mitochondrial membrane (OMM); outer mitochondrial membrane (Omm); S-adenosylmethionine (AdoMet); S-cysteine (Cys); methionine (Met); methionine (MM); cysteine (Met).

species but different from those of DNA ligases I, III and IV (Waterworth et al., 2010). In desiccated seeds, DNA damage is repaired on the initiation of imbibitions that is perceived as a promising phenomenon in enhancing life tenure and germination percentage of seed (Balestrazzi et al., 2011). Waterworth et al. (2010) reported that a mutant of the plant-specific DNA ligase 6 gene (AtLIG6) exhibited a delayed seed germination phenotype. Protein primary structure analysis revealed that the  $\beta$ -CASP motif of the AtLIG6 N-terminal region is present in a number of repair enzymes, and moreover, double mutants of AtLIG4 and AtLIG6 and single mutants of AtLIG6 have been found to possess noteworthy hypersensitivity to seed aging and to exhibit reduced viability and delayed germination, compared to wild-type plants. These findings confirm that AtLIG6 is a main regulator of seed quality and longevity in *Arabidopsis*. In *Medicago truncatula*, the enzymes that repair oxidized DNA (e.g., formamido-pyrimidine-DNA glycosylase and 8-oxoguanine DNA glycosylase/lyase) are upregulated during the initial stages of germination, which supports the importance of DNA repair during seed germination (Macovei et al., 2011). Moreover, the *Arabidopsis* knockout mutant *nbs1* has verified that DNA repair subunit NBS1 controls telomeric homeostasis. Furthermore, until now, no signs of developmental aberrations have been noted in NBS1-deficient plants. However, they produce fertile and viable seeds in subsequent generations upon self-fertilization (Najdekorva and Siroky, 2012), and intense developmental defects have been reported in double-mutant plants missing the functional telomerase till three generations. Cytogenetic inspection and molecular methods employed for the examination of telomere length in double homozygous mutants revealed a much rapid telomere contraction than in plants lacking telomerase gene. These observations confirm that *nbs1* and telomerase unanimously play vital roles in plant telomere renewal, which can also be important for seed viability. Nicotinamide functions as an inhibitor of poly (ADP-ribose) polymerases, which regulate DNA repair process. Therefore, it should be degraded during germination. The expression analysis of the nicotinamidase-encoding gene NIC2 in *Arabidopsis* has revealed that NIC2 is expressed at relatively higher rates in mature *Arabidopsis* seeds (Hunt et al., 2007). This is further supported by the analysis of an *Arabidopsis* NIC2-1 knockout mutant, in which seeds have diminished nicotinamidase activity and hampered germination. This observation suggests that NIC2 metabolizes

nicotinamide, thus, accelerating poly(ADP-ribose) polymerases activity and initiating the DNA repair process (Hunt et al., 2007).

#### *Enzyme repair*

The aging process in living organisms is related to DNA damage, as well as protein dysfunction. Spontaneous damage of proteins during cell aging is attributed to the generation of abnormal amino acid residues. The protein L-isoaspartyl methyltransferase (PIMT) catalyzes the transformation of abnormal L-isoaspartyl and L-isoasparaginyl residues to their normal L-aspartyl and L-asparaginyl forms, thus mitigating protein misfolding owe to isomerized aspartyl (Asp) and asparaginyl (Asn) residues formation. Moreover, the proteins of a variety of organisms are spontaneously enriched by residual accumulation of isomerized aspartyl and asparaginyl residues. Therefore, the PIMT repair enzyme system plays a vital role in the survival and longevity of organisms in the animal and bacterial kingdoms (Clarke, 2003). However, altered PIMT1 expression in *Arabidopsis* lines validates its role as a main endogenous dynamic controlling anomalous deposition of L-isoaspartyl in seed proteins, thus, enhancing seed vigor and longevity (Oge et al., 2008). Other anti-ageing pathways and PIMT repair pathway work unanimously to eradicate hazardous protein outcomes, thus ensuring the successful establishment of seedlings. Recently, PIMT2 was reported to have a similar function as PIMT1 in both *Arabidopsis* and chickpea. The major difference is their localization, with PIMT1 functioning in the cytosol and PIMT2 in the nucleus (Verma et al., 2013). In dehydrated orthodox seeds, proteins spontaneously degrade into succinimide derivatives of Asp and Asn residues, which are isomerized upon imbibition into derivatives of isoAsp (Nayak et al., 2013), which is deleterious to appropriate protein function and, if unrepaired, will compromise seed vigor. Although PIMT mediates isoAsp transformation to Asp residues, proteins associated with translation upon imbibitions in orthodox seeds are still critically important to sustain the seeds in the active state. PLANT RNA HELICASE 75 (PRH 75), which is also known as Dead-box helicase, exhibits susceptibility to isoAsp residual deposition, as traced in *Arabidopsis thaliana*. This confirms that PIMT repairs PRH75 via isoAsp conversion to Asp residues, coupled with seed vigor restoration. In most plant species, seeds possess reduced PIMT activity during germination. However, consistent PIMT activity in sacred lotus seeds minimizes the racemization of aspartyl in proteins; therefore, they possess exceptional longevity (Shen-Miller, 2002).

### **Epigenetic regulation and telomere length**

Epigenetics demonstrates heritable variations in genes expression or cellular phenotypes without any change in sequential architect of DNA. Epigenetic modulation of gene manifestation is destined by histone variants, histone modifications, chromatin remodeling, DNA methylation, and may involve small RNAs (Ahmad et al., 2010). In recent years, epigenetics has become one of the most exciting areas in functional genomics. In higher plants, seed germination and vigor are associated with the expression of a great number of genes and are related to various epigenetic processes.

#### *DNA methylation*

In genomic DNA, methylation of fifth carbon of cytosine (5-methylcytosine) is frequently linked with heterochromatic loci comprising centromeres and telomeres and is also concurrent with various silent loci, especially transposable elements and repetitive sequences (Lu et al., 2006; Le et al., 2007; Bauer and Fisher, 2011). The involvement of DNA methylation in the seed germination process was confirmed through methylation-sensitive amplification polymorphisms (Portis et al., 2004; Meng et al., 2012).

In the seeds and seedlings of pear (*Pyrus communis*), DNA methylation has been reported to increase as the moisture level declined 2.3%, whereas DNA methylation was reduced when the moisture content increased to 5.3% (Michalak et al., 2013). Moreover, significantly increased DNA methylation has been reported in seeds under storage period of one year (Singh et al., 2013). These results suggest the possibility of seed aging coupled with increased DNA methylation. The inhibitory effects of DNA methylation on seed germination have also been reported in *Arabidopsis*, in which JMJ20 and JMJ22 (histone arginine demethylases) serve as redundant positive mediators of germination. The zinc finger-protein SOMNUS directly represses JMJ20/JMJ22 during the inactivation period of phytochrome B (PHYB). However, a boost in gibberellic acid level is detected during PHYB activation and JMJ20/JMJ22 depreciation, as a result of the excision of repressive histone arginine methylations from GA3ox1/GA3ox2, which accelerates the germination process (Cho et al., 2012). More recently, it has been disclosed that the modulated DNA methylation of two wheat genes (i.e. AGO802 and AGO804), which are differentially expressed in the embryos of developing seeds, were related to pre-harvest sprouting (Singh et al., 2013). Along with DNA

methylation, histone methylation is also of pivotal importance to seed viability. Moreover, epigenetic states catalyzing histone H3 lysine 27 trimethylation (H3K27me3) being a repressive chromatin mark has polycomb repressive complex (PRC2) as a key regulator. Bouyer et al. (2011) reported that transitions from embryonic to seedling phases and from vegetative to flowering phases are controlled by robust PRC2-mediated regulation via histone methylation. Müller et al. (2012) also confirmed the function of PRC2 and H3K27me3 in seed germination. The authors reported that this mode of action, histone methylation/demethylation, together with ABI3, was evolutionarily conserved from gymnosperms to angiosperms. A significant feature of aging is associated with telomeres, the repetitive sequence of nucleotides situated at each terminus of chromatid, which protects the terminus from deterioration or from fusion with adjacent chromosomes, thus preserving genomic integrity. Likewise animal cells, dynamicity of plant telomeric DNA determines morphological variations during aging and differentiation (Kilian et al., 1995). Despite having identical germination rates, significant differences in telomeric lengths were reported in both fresh and stored wheat seeds (Bucholc and Buchowicz, 1992; Bucholc and Buchowicz, 1995). Various studies have reported a strong association between aging and telomere length, as indicated by the breakdown of telomeric sequences (Boubriak et al., 2007). Dona et al. (2013) also found that the telomeric length of seeds from two alpine species, *Silene acaulis* and *Silene vulgaris*, decreased and increased significantly upon drying and rehydration, respectively. Under artificial aging conditions, seeds of both species exhibit significant reductions in telomere length. Moreover, in the chromosomes of dry seeds, the restoration of telomere ends occurs through the initiation of telomerase activity (Riha et al., 1998). Balestrazzi et al. (2010) suggested that increases in telomere length at the initial stage of imbibition are associated with seed repair processes, which are catalyzed to eliminate DNA fragments. Moreover, under long-term storage, dried wheat embryos were devoid of telomeric repeats. However, fortification was observed at the early stage of imbibition (Bucholc and Buchowicz, 1992). There are certain reports that describe the impact of fluctuated telomeric homeostasis on seed vigor and viability, which may validate the link between genotoxicity and increased telomeric length. For example, impaired seed germination was reported in the *Arabidopsis rtbp1* (rice telomere binding protein1) mutant, owing to increased telomeric length, as compared to wild-

type plants (Hong et al., 2007). Similarly, an *Arabidopsis* mutant lacking ku70 function, which is required for telomere maintenance, exhibited increased telomere length, with a high susceptibility to genotoxicity (Riha et al., 2002). Currently, a framework is devised in plant and animal cells, where exogenous or endogenous oxidative damages to telomeres can be distinctly identified, owing to differentially induced responses (Wang et al., 2010). Therefore, it can be concluded that damages resulting from extrinsic factors contribute to telomere degradation. However, moderate injuries caused by cellular metabolism promote telomere lengthening. This model can be applied to seeds where telomeric degradation might be associated with artificial aging, whereas increased telomere length might be expected after rehydration, as a result of resumed metabolic processes.

#### *Micro RNA (miRNA) and argonaute (AGO)*

Recently, small RNAs, particularly miRNAs, have become a key research area in molecular biology and genomics. miRNAs of 20-24 nucleotides in length are usually transcribed from non-coding regions of the genome. RNA polymerase II transcribes precursors yield single-stranded precursors upon excision are known as miRNAs. These precursors construct stem-loop secondary structures, owing to the presence of self-complementary regions that are recognized by DCL1 proteins and converted into miRNA:miRNA\* (Park et al., 2002). Moreover, ARGONAUTE (AGO) protein associates with miRNA in duplex to form RNA induced silencing complex, where miRNA base pairs with complementary sequence in target mRNA to direct its post-transcriptional regulation via translational repression or cleavage (Jeong et al., 2013). The involvement of miRNAs in the regulation of gene expression has been known for many years; however, their regulatory function at translational levels has only recently been discovered (Li et al., 2013). Martin et al. (2005, 2006) first described the function of miRNA in *Arabidopsis* seeds. miRNAs 159 and 160 are thought to be involved in seed germination (Reyes and Chua, 2007; Liu et al., 2007; Table 2).

Various AGO genes from *Arabidopsis* (10) and rice (19) have been reported to function as effectors in RNA-induced silencing, but their function in plants, particularly in seeds, largely remains unclear (Rogers and Chen, 2013; Yang et al., 2013). Mallory and Vaucheret (2010) summarized the expression mode of AGO genes in *Arabidopsis*. The authors suggested that AGO1, AGO2, AGO3, AGO5, AGO6,

AGO7, AGO9, and AGO10 are significantly expressed in seeds, and Yang et al. (2013) reported that, during rice seed germination (72 h after imbibition), AGO1a, AGO1b, AGO1c, AGO2, AGO4a, AGO4b, AGO7, AGO16, AGO18, and PNH1 were significantly expressed, with AGO4b, AGO4a, and AGO1a exhibiting the highest expression levels.

Recently, two AGO genes (*TaAGO1b* and *TaAGO4*) were cloned and characterized from common wheat (Liu et al., 2011; Meng et al., 2013). Interestingly, the genes were only differentially expressed in the endosperm during germination and were ubiquitously expressed in embryonic tissues. Similarly, in the endosperm of germinating wheat seeds (6 h after imbibition), the transcript level of *TaAGO4* was reported to decline, which suggests the possible involvement of miRNA in seed germination and viability (Meng et al., 2013).

#### **Genomic tools for monitoring seed aging**

The above-mentioned information indicates that currently available methods in cell biology, biochemistry, and genomics can be used to monitor seed aging at various stages. Genomics (including functional genomics) has played a major role in current biological research (Kujoth et al., 2005). In contrast to genetics or molecular biology, which focuses on individual genes, genomics allow researchers to investigate the structure and function of entire gene families and genomes. Due to the rapid progress in plant genomics, large numbers of genes and regulators have been revealed to be associated with seed germination and viability (Ding et al., 2013). Therefore, the new knowledge from genomics can be used for the development of seed biotechnology. Furthermore, some new technologies, such as next generation sequencing (NGS), are particularly efficient for studying seed deterioration.

Next-generation sequencing technology, including 454, Illumina, and SOLiD, has recently become a key approach in molecular biology and genomics. The technology is high throughput, low cost, reliable, and precious qualification (Shah et al., 2017) and can be used for characterizing genomic DNA, mitochondrial DNA, mRNA, and small RNA (Ansorge, 2009). So far, NGS has been reported to provide better assessments of seed deterioration (Chaudhury et al., 2001; Fu and Peterson, 2012), and to the best of our knowledge, NGS technology can be utilized in the following types of research.

### Transcriptomics

Seed aging is coincident with transcriptome alternation, which can be investigated using NGS, and the effects of seed priming and stress treatment can be identified by characterizing the corresponding transcriptomes.

### Mitochondrial genomic alternation

Mitochondrial genomes are much smaller than nuclear genomes. When mitochondrial genomes are exposed to ROS, mitochondrial genome alternation has been proposed to contribute to seed aging. Thus, it is logical to consider using NGS to investigate mitochondrial genome alternation in aging seeds.

### miRNA transcriptomics

Quite a few types of miRNA are involved in the process of seed germination. Using NGS, the alternation of miRNA transcriptomes in aging seeds can be clearly identified (Table 2).

### Genomic methylation analysis

The methylation state of a whole genome, or methylome, has been measured at single-base

resolution using bisulfite conversion of genomic DNA, combined with NGS (BS-seq; Krueger et al., 2012). This strategy could also be used to monitor seed aging in the future, especially for the mitochondrial genome (Lu et al., 2006; Birtic et al., 2011). DNA methylation has been implicated in the seed germination process, using methylation-sensitive amplification polymorphisms (Portis et al., 2004; Meng et al., 2012). Therefore, to characterize DNA methylation during seed aging, methylation-sensitive amplification polymorphisms may serve as a new tool. From the polymorphic data of DNA methylation from aging seeds, we can measure seed viability.

Seed aging can also be assessed by measuring genomic DNA methylation via investigation of the genome's global m5C content, which can be accomplished using thin-layer chromatography or high pressure liquid chromatography (HPLC). However, thin-layer chromatography is more feasible, since a smaller amount of DNA is needed to differentiate ribo- and deoxyribonucleotides under experimental analysis (Michalak et al., 2013).

**Table 2.** Summary of miRNAs related to seed germination and post germination transition.

miRNA	Target Gene	Plant Source	Authors and Year
miR156 miR156a	SPL13, SBP domain protein,	<i>Arabidopsis</i> , <i>Lotus Japonica</i> Maize, <i>Brassica napus</i>	Martin et al., 2010 ; Hu et al., 2013 ; Ding et al., 2012 ; Li et al., 2013 ; Huang et al., 2013
miR159	MYB33 and MYB101,	<i>Arabidopsis</i> , Maize	Reyes and Chua, 2000; Li et al., 2013
miR160 miR160a	ARF10	<i>Arabidopsis</i> , <i>Lotus Japonica</i>	Liu et al., 2007 ; Hu et al., 2013
miR164	NAC1	Maize	Ding et al., 2012 ;Li et al., 2013
miR166	HD-ZIP	Maize	Li et al., 2013
miR167	ARF	Maize	Li et al., 2013
miR168	Argonaute1 (At1g48410)	<i>Arabidopsis</i> , Maize	Ding et al., 2012 ; Li et al., 2013 ; Baldrich et al., 2014
miR169	NFY,	Maize	Ding et al., 2012 ; Li et al., 2013
miR172	SNZ;	<i>Arabidopsis</i> , Maize	Martin et al., 2010 ;Li et al., 2013
miR319	MYB,	Maize	Li et al., 2013
miR393	TIR1-like,	Maize	Li et al., 2013
miR394	LCR	Maize; <i>Arabidopsis</i>	Li et al., 2013 ; Song et al., 2016
miR395c; miR395e	APS	<i>Arabidopsis</i>	Kim et al., 2010
miR396	GRFs,	Maize	Ding et al., 2012
miR397	LCR	Maize	Li et al., 2013 ;
miR398	?	Maize	Li et al., 2013
miR399a	?	<i>Lotus Japonica</i>	Hu et al., 2013 ; Song et al., 2016
miR408	plantacyanin Cupredoxin	<i>Arabidopsis</i> , Maize	Li et al., 2013 : Ding et al., 2016
miR528	CSD/CPD;	Maize	Li et al., 2013
miR529	?	Maize	Li et al., 2013

?; Not reported

Moreover, qRT-PCR has been used to study the transcript level of some regulatory and functional genes involved in seed germination (Liu et al., 2011; Feshani et al., 2012; Hu et al., 2013; Meng et al., 2013). The alternation of transcript levels of some germination-required genes in aging seeds can be detected using qRT-PCR. Thus, some sensitive genes, e.g., AGO genes, miRNA, anti-oxidative genes can serve as biomarkers for seed aging by using qRT-PCR.

As described above, DNA repair (Waterworth et al., 2010; Najdekorva and Siroky 2012), enzyme repair (Oge et al. 2008; Nayak et al., 2013), and anti-oxidation genes (Yao et al., 2012) are all reported to influence seed viability. In order to develop new tools for monitoring seed aging, qRT-PCR can be used to inspect the expression level of related anti-oxidative genes (Dona et al., 2013). Recently, Hu et al. (2012) reported that controlled deterioration treatment immediately invokes significant increases in caspase-3/DEVDase proteolytic activity. Therefore, qRT-PCR can also be used to detect the expression of the protein in stored seeds.

In addition, since the early of 1990's, telomere shortening has been implicated in cell aging in almost all types of living organisms, including plants (reviewed by Watson and Riha, 2011). Seed aging was assessed effectively by measuring the length of telomeres in seeds (Boubriak et al., 2007; Dona et al., 2013). Therefore, telomeric investigation has been proposed as a reliable marker of seed aging, and both Southern blotting and qRT-PCR have been used for telomere length analysis (Boubriak et al., 2007; Dona et al., 2013).

#### *Molecular markers*

DNA alternation has been reported to trigger PCD during seed deterioration (Liu et al., 2005; Hu et al., 2012). The two basic strategies used to detect DNA alternation efficiently are the identification of DNA laddering, using electrophoreses, and the identification of DNA alteration, using RAPD markers. In various studies, the use of RAPD has been recommended as an appropriate procedure to detect DNA damage and mutational events, including point mutations, insertions, structural rearrangements, deletions, and other possible changes, such as structural distortion (Atienzar and Jha, 2006; El-Maarouf-Bouteau et al., 2011). Quantitative trait loci associated with seed longevity have been thoroughly investigated, in order to elucidate the genetic basis of seed deterioration (Shatters et al., 1995; Arif et al., 2012). As a result of

these efforts, genes that govern seed longevity have been identified and may facilitate the prediction of the longevity in plant germplasm repertoires (Nagel et al., 2009; Vijay et al., 2009). For example, four genomic regions associated with wheat seed longevity are known to include genes related to stress responses and spike traits (Arif et al., 2012). These outcomes are encouraging, since the prediction of seed viability will facilitate the improvement monitoring of stored seeds.

To fully utilize the increasing amounts of high-quality genome-wide transcriptomic data associated with seed viability and aging, novel analytical tools are needed. For example, the co-expression analysis approach, which was used in *A. thaliana* to undermine gene functions engaged in the mediation of seed germination (Bassel et al., 2011a), can also be applied to the functionality of genes involved in the regulation of seed aging. Along with co-expression analysis, Bassels et al. (2011b) recently proposed that co-prediction analysis, which allows the prediction of functional gene associations that would not be identified via co-expression analysis. With co-prediction analysis, microarray data set under different physiological conditions, such as conditions under storage and germination, can be used. Conventional transcript analysis has continuously failed to detect specific coordination during aging regulated by common *cis*-elements and *trans*-factors; however, co-prediction could be an effective tool (Bassel et al., 2011b). During the aging of pea seeds, PCD is associated with changes in the half-cell reduction potential of the major cellular antioxidant and redox buffer glutathione [E(GSSG/2GSH)] (Kranner et al., 2006). Similarly, Birtic et al. (2011) hypothesized that the half-cell reduction potentials of low molecular weight cysteine, thiols, g-glutamyl-cysteine, and cysteinyl-glycine can be potentially used as markers for seed aging (Table 3).

#### **Prospects**

Although seed deterioration is an active area of research, its complex dynamics are poorly understood. Seed aging is a complex physiological process. It is difficult to search for signals from various stages to predict the status of seed viability. This review reveals several interesting signals that could be utilized to monitor seed viability. These signals include ROS- and mitochondrial-triggered PCD in aging seeds, the transcription of anti-oxidative and DNA and protein repair genes, telomere length, the expression of epigenetic regulation-related genes (miRNA and methylation),

**Table 3.** Summary of functional genomic marker genes related to seed viability.

<b>Gene</b>	<b>Functional Protein</b>	<b>Plant Source</b>	<b>Authors</b>
<i>APX</i>	Ascorbate peroxidase	Pea, <i>Triticum aestivum</i>	Yao et al., 2012 ; Padaria et al., 2014
<i>CAT</i>	Catalase	Pea	Yao et al., 2012
<i>GR</i>	Glutathione reductase	Pea, <i>Arabidopsis</i>	Yao et al., 2012; Yu et al., 2013 ; Ding et al., 2016.
<i>SOD</i>	Superoxidate dismutase	Pea, <i>Silene vulgaris</i> , <i>Silene acaulis</i>	Yao et al., 2012; Dona et al., 2013
<i>MT2</i>	Type 2 metallothionein	Pea, Soybean, <i>Silene vulgaris</i> , <i>Silene acaulis</i>	Dona et al., 2013
<i>Atlig6</i>	DNA ligase VI	<i>Arabidopsis</i>	Watsworth et al., et al., 2010; Furukawa et al., 2015
<i>NBS1</i>	DNA repair subunit on telomere	<i>Arabidopsis</i>	Oge et al., 2008 ; Najdekorva and Siroky, 2012
<i>PIMT1</i>	L-isoaspartyl methyltransferase	<i>Arabidopsis</i>	Nayak. et al., 2013 ; Verma et al., 2013
<i>PIMT2</i>	L-isoaspartyl methyltransferase	<i>Arabidopsis</i> , Chick-pea, <i>Silene vulgaris</i> , <i>Silene acaulis</i>	Dona et al., 2013
<i>At3g08030</i>	cell-wall-associated protein	<i>Arabidopsis</i> , <i>Ceiba aesculifolia</i> , <i>Wigandia urens</i>	Garza-Caligaris et al., 2012

and alternations in mitochondrial and nuclear genome sequences. Among these approaches, genomic tools may be more effective, since genomic signals can be detected directly and clearly, whereas PCD analysis, such as TUNEL, is time consuming, complex, and indirect.

It seems that NGS has opened many avenues to assess the genetic changes that occur during seed development and exhibits the most promise for the development of effective tools to monitor seed viability. Next-generation sequencing can be used to investigate the transcriptome of aging seeds, which can elaborate the multiple aging related genomic signals (including anti-oxidative genes, DNA and enzyme repair genes, miRNA related genes, and telomeres), and can be used to analyze sequence mutations in both mitochondrial and nuclear genomes, as well as epigenetic process, by characterizing small RNA and methylation alteration in aging seeds. For all these, the cost of NGS is much lower than that of Sanger sequencing and provides much more information. Therefore, searching for effective tools to monitor seed viability may provide advances in the assessment of seed viability and provide a useful supplement to the

traditional methods of germination testing for the long-term conservation of *ex-situ* plant germplasm.

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