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Review

An Insight into the Behaviour of Recalcitrant Seeds by Understanding Their Molecular Changes upon Desiccation and Low Temperature

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Abstract: Systems biology is an interdisciplinary study that involves a combination of expertise in biology, chemistry, mathematics, physics, and engineering to unravel the biology of complex living systems by incorporating multiple kinds of quantitative molecular computations by using sophisticated mathematical models. This interdisciplinary study can be applied to identify and understand molecular and metabolic changes in recalcitrant plant species. Many tropical plants with recalcitrant seeds have difficulty with long-term seed storage and preservation due to their intolerance to desiccation and low temperatures. The aim of this review was to explore and discuss how omics analyses can assist in elucidating molecular responses and metabolic changes of recalcitrant seed species. Genomics and transcriptomics analyses identified genes, such as late embryogenesis abundant (LEA), that were highly expressed after exposure to desiccation and low temperatures. Meanwhile, proteomic analysis using 2D gel electrophoresis, MALDI-TOF MS, or MS/MS analysis revealed dehydrins induced from recalcitrant seeds upon exposure to desiccation and low temperatures. Metabolomic analysis using liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) profiling of recalcitrant seeds has discovered metabolites such as sugar and organic acid changes in recalcitrant seeds at different developmental stages. This information may contribute to comprehending the behaviour of recalcitrant seeds and provide insight into how crop management can be improved in terms of seed storage for conservation in order to maintain plant biodiversity.

Keywords: desiccation sensitivity; omics analysis; recalcitrant seed; seed storage

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1. Introduction

Seed science and technology are key for sustaining the seed's physical, physiological, and genetic quality. The viability of seeds in storage varies due to differences in tolerance acquisition. Seeds can be categorized into three main groups: orthodox, intermediate, and non-orthodox, which is mainly known as recalcitrant [1]. Storage longevity varies among these three seed categories. Orthodox seeds with low water content acquire desiccation

tolerance during development and can be stored dry for longer periods of time; they can be preserved under ambient conditions for 5–10 years of half-life time. Meanwhile, they can be preserved 40–60 years under more optimal conditions [2,3], whereas recalcitrant seeds with high water content are difficult to store for long periods with the same conditions as orthodox seeds because they can quickly lose their viability [4]. Apart from orthodox and recalcitrant seeds, there is another category: intermediate seed. Intermediate seeds are in between orthodox and recalcitrant; they can be stored longer than recalcitrant seeds but not as long as orthodox seeds [5]. However, even though the seed can be classified as orthodox, recalcitrant, or intermediate, this has not always been the case. The *citrus* seed has been considered as a recalcitrant species for a long time [6], but initial studies on *citrus limon* seeds revealed that the species demonstrated desiccation tolerance [7]. Meanwhile, it was determined that other *citrus* species could not be stored under conventional conditions with a 3–7% water content, which revealed that it bears recalcitrance behaviour [8]. Therefore, further investigation and comparison among species should be conducted for other seed species in order to determine if there is any variation in terms of their desiccation tolerance or sensitivity.

The crucial factors determining seed longevity are water content, temperature, and relative humidity [9]. Any changes that occur to the seed water content may impact the storability and longevity of seeds [10]. Hence, maintaining seed viability is vital as the seed quality can significantly affect the uniformity of development (germination and dormancy), yield, and quality of the harvested crops. Furthermore, food security and crop production depend on maintaining the seed's quality and viability after storage (in seed banks). An excessive loss of water content or drought, exposure to extreme temperatures (high and ultra-low), and other abiotic stresses such as salinity, light, and high metal toxicity greatly impact seed viability [11]. These stresses that persist in plants may promote metabolic responses that will regulate their growth and development, as well as for survival in harsh environmental conditions by producing a vast variety of flexible and adaptable regulators [12]. For example, the water content for storing six different Brassicaceae species ranged from 2–3% [13]. Interestingly, for a variety of species, the critical water content of orthodox and recalcitrant seeds overlaps between 20 and 30% [14].

Generally, orthodox seeds that are stored in a seed bank are dried to 5–6% water content and placed in sealed containers stored at temperatures below −8 °C [15]. For effective seed storage and conservation, the temperature and relative humidity must also be considered thoroughly to conserve seeds effectively [16]. Moreover, seeds are dried to equilibrium, which is conducted in a controlled environment of 5–20 °C and 10–25% relative humidity depending on the species prior storing from −18 °C to −20 °C [2]. Therefore, the minimum requirement for storing seeds in a seed bank is that seeds should be kept under controlled conditions, ensuring that the accession's viability remains above at least 85% from 10 to 20 years [15].

Previous reviews proposed systems biology application for identifying potential factors in resolving recalcitrant seed-storage difficulties [17]. However, omics data regarding recalcitrant seeds are still lacking. Understanding the molecular responses towards biotic and abiotic stresses at the level of DNA-based markers, genomics, epigenomics, transcriptomics, proteomics, and metabolomics was discussed on the recalcitrant species, but only on *Quercus ilex* [18]. Therefore, this review provides insight into genes, proteins, and metabolic changes of several other recalcitrant seed species using a systems biology approach. The advancement of high throughput tools via omics analyses can assist in understanding the mechanisms that are involved in recalcitrant seeds when they undergo stresses like desiccation and ultra-low temperatures. Further studies are suggested, as well as using systems biology approaches to provide a deeper understanding on the recalcitrance behaviour of the molecular prospects.

Some reviews on recalcitrant species utilizing omics analysis have previously been published by authors' research groups [17,18]. In this review, Table 1 shows there are several papers found in the Web of Science (WOS) and Google Scholar database, which

report on the use of omics approaches on several recalcitrant species. These reports may provide comprehensive information of the molecular mechanisms of recalcitrant species. This involves analysis from all levels, ranging from genes to metabolites through omics analysis in order to gain a deeper understanding.

Table 1. Number of papers found in the Web of Science (WOS) and Google Scholar database (21 March 2023) from a search using the species name and the omics approach employed as the keywords.

Species	Genomics	Transcriptomics	Proteomics	Metabolomics	References
Garcinia sp.	2	1	-	3	[19–24]
Camellia sp.	-	2	1	-	[25–27]
Quercus sp.	-	1	-	1	[28,29]
Panax sp.	-	1	-	-	[30]
Ocotea sp.	-	-	1	-	[31]
Castanospermum sp.	-	-	1	-	[32]

1.1. Comparison between Orthodox and Recalcitrant Seeds

Orthodox seeds are seeds that can acquire desiccation tolerance during development and are able to be stored with low moisture and temperatures for a long period of time. Orthodox seeds should possess high vigour and viability, at least from the harvest until the next growing season, or for many decades at −18 °C [3]. In addition, orthodox seeds would undergo a period of drying during their maturation and are shed at a low water content, which is in equilibrium with the prevailing relative humidity. The equilibrium water content at any particular relative humidity is determined by seed composition. All orthodox seeds can withstand dehydration to around 5% (0.053 g H_2O g^{-1} dry material [g g^{-1}]), even when maturation drying is not completed prior to shedding time [3].

Differences between long-lived (orthodox) and short-lived (non-orthodox/recalcitrant) species can be identified based on their size and water content. Examples of crop plants that bear orthodox seeds are wheat (*Triticum aestivum* L.), cereal rye (*Secale cereale* L.), and legumes such as the common bean (*Phaseolus vulgaris* L.), rice (*Oryza sativa*), banana (*Musa balbisiana*), and guava (*Psidium guineense*) [2,33–35]. Apart from their capability to retain viability when dried to a low water content of 2−5%, orthodox seeds can be also distinguished from recalcitrant seeds based on their size, which is relatively smaller than recalcitrant seeds, as shown in Figure 1 [36]. Rapeseed plants (*Brassica napus oleifera* L.) bear orthodox seeds, which include sizes ranged from 1.5–2 mm [37]. In addition, paddy plants, which also bear orthodox seeds, have seed lengths ranging from 8.61–11.29 mm [38]. However, recalcitrant seeds, such as *Garcinia* species, have seed lengths ranging from 1.1–2.5 cm [39]. Moreover, large recalcitrant coconut (*Cocos nucifera* L.) seeds include lengths ranging from 19.3–27.8 cm [40].

The ability of orthodox seeds to survive desiccation is related to the induction of defensive mechanisms during dehydration, including the accumulation of non-reducing sugars, polyols, amino acids (i.e., proline), late embryogenesis abundant (LEA) proteins, and heat-shock proteins [41]. When cells lose water during dehydration, cytosolic components become more concentrated. The late embryogenesis abundant (LEA) protein gene family is essential for cellular defence mechanisms as a response to abiotic stress [42]. The LEA genes are primarily expressed in seeds that accumulate during the late embryonic development stage [43]. The non-reducing sugars and LEA proteins form intracellular glass, which then reduces the molecules' uptake across the cytoplasm and eventually limits chemical reactions [10]. Intracellular glass is also known as cytoplasmic glass or vitrified cytoplasm. It is a phenomenon observed in certain plant seeds during desiccation. It refers to the transformation of the cytoplasm within the cells into a glassy or amorphous state when the seed is subjected to extreme dehydration [44].

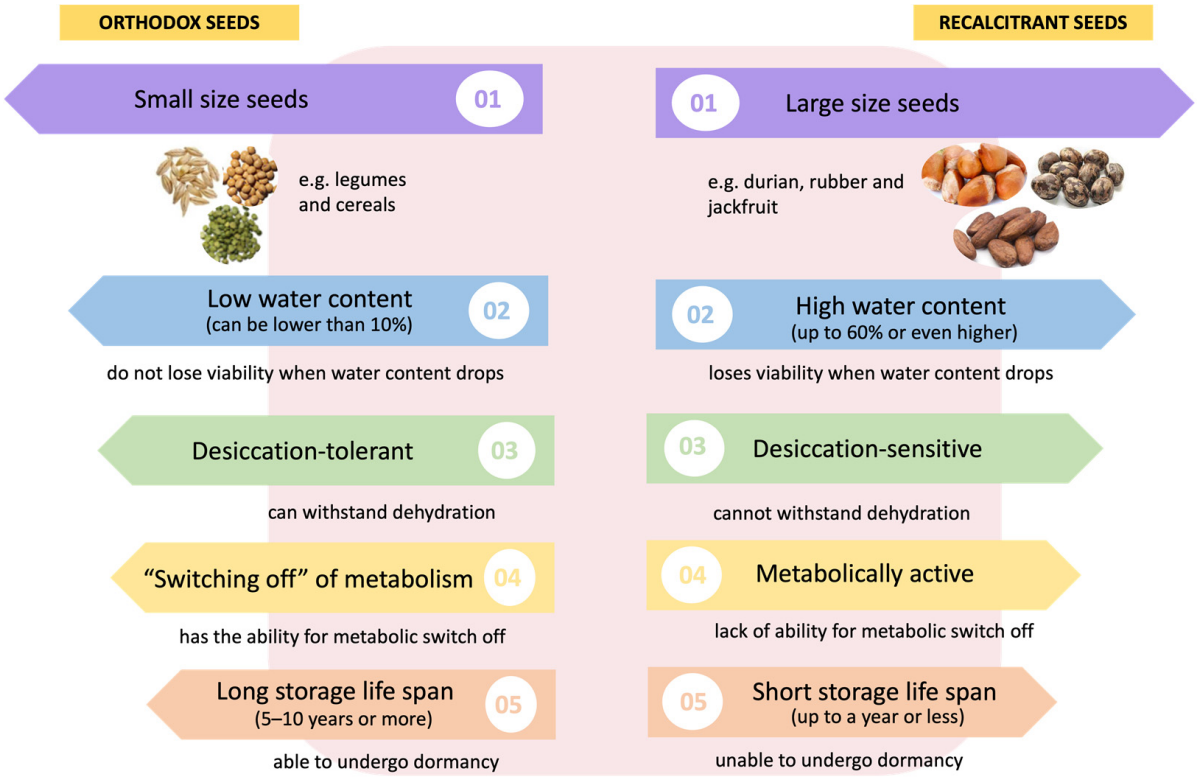

Figure 1. Comparison between orthodox and recalcitrant seeds that contribute to their distinct storage behaviours.

As a result, the seeds become dormant and remain stable for long periods. However, non-orthodox seeds or recalcitrant seeds are sensitive to desiccation. Unlike orthodox seeds, species with recalcitrant seeds are metabolically active and cannot undergo dormancy under unfavorable conditions to germinate [45]. For this reason, recalcitrant species have far shorter lifespans and less viability than other orthodox species.

Recalcitrant seeds contain high water content and will lose their viability when the water content drops. For instance, recalcitrant seeds of mangosteen (*Garcinia mangostana*), a well-known tropical plant for its xanthone content, lose their viability and ability to germinate when the water content drops to 30% or lower [39,46]. Other examples of tropical plants that bear recalcitrant seeds are durian (*Durio zibethinus*), with 54.9% water content [47], rubber (*Hevea brasiliensis*), with 45.73% water content [48], and cacao (*Theobroma cacao*), with 66.6% water content [49].

Indeed, many tropical plants from tropical regions produce recalcitrant seeds [45]. Recalcitrant seeds are typically huge, with a short lifespan and high water content [50]. These seeds are metabolically active right after shedding from the mother plant. Due to the high water content, recalcitrant seeds cannot withstand drying and freezing during storage [51], as ice crystallization will form. Subsequently, recalcitrant seeds undergo cellular damage, germinate [41], and lose seed viability.

There are a number of challenges and problems that emerge when dealing with recalcitrant seeds. For instance, seed storage for ex situ conservation becomes difficult as it is a long-term storage, which is not suitable with short-lived recalcitrant seeds [52]. Furthermore, species with recalcitrant seeds usually possess a slow growth rate and fruit yield. As a consequence, the large-scale cultivation of this species for crop production would be inefficient [53].

Furthermore, seed viability can easily decrease when water content drops to a certain percentage [16]. Specifically, recalcitrant seeds can lose their viability when it declines to 24–35.5% [46]. Moreover, there is an absence of a maturation-drying phase as seeds

are metabolically active during their development [54]. Many tropical crops, such as coconut (*Cocos nucifera*), durian (*Durio zibethinus*), and mangosteen (*Garcinia mangostana*), have recalcitrant seeds that make propagation and conservation difficult. Moreover, there is an absence of the maturation-drying phase as seeds are metabolically active during their development [54]. Hence, in vitro propagation has been implemented as one of the solutions to combat this hindrance [50]. Furthermore, there is a cryopreservation method, which is an alternative to conventional seed bank storage for recalcitrant seeds by using liquid nitrogen at −196 °C [41]. Cryopreservation is a more promising alternative for storing recalcitrant seeds because this method utilizes specific protocols and cryoprotectants to minimize ice crystal formation, which helps prevent cellular damage and preserve the integrity of biological samples [1].

1.2. Mechanism and Metabolic Changes in Seeds upon Stress

1.2.1. Reserves Accumulation

Reserves or storage compounds such as carbohydrates, proteins, and oils can be accumulated abundantly by seeds which then serve as the main source of nutrients to initiate seed germination. In addition to acting as reserves, the production of these compounds act as the plant's mechanism in response to biotic and abiotic stresses. Plants synthesize various classes of sugars and sugar alcohols as a response to desiccation [55]. For example, sucrose, raffinose, stachyose, and cyclitols were accumulated in seed tissues of *Inga vera*, *Caesalpinia echinate*, and *Erythrina speciosa* after being subjected to different levels of drying [56]. Interestingly, those compounds were discovered to have higher concentrations in desiccation-tolerant *C. echinate* and *E. speciosa* than in recalcitrant *I. vera* [56]. Sugars and sugar alcohols were found to not only act as osmoprotectants but also as antioxidants in response to oxidative stress caused by environmental changes [57]. Trehalose was observed to accumulate in the embryonic axis of *E. speciosa*, suggesting that it may have a significant impact on desiccation tolerance [58]. The compound accumulations indicate a substantial relationship between seeds' storage behaviour and sugar metabolism, and they help to increase seed resistance to drying and freezing [59,60]. Additionally, trehalose is considered to be involved in the vitrification of seeds, which slows down enzymatic activities and helps to protect seeds' membranes from damage and cellular component deterioration [61].

In addition to carbohydrate accumulation acting as storage compounds, oil content and lipids also serve as reserves in seeds. Studies conducted on soursop (*Annona muricata*) seed oil determined that the the average polyunsaturated fatty acid (PUFA) content varied from 31.72% in sundried seeds to 30.92% after 30 h of oven-drying; the drying process did not have a significant impact on the fatty acid content [62]. However, slow drying conducted on the intermediate seed and coffee seed (*Coffea arabica*) showed a significant increase of the concentration of free fatty acid (FFA) content [63]. These findings explain that the concentration of the accumulation of oil content and lipids as reserves in seeds may vary based on the seed category and the type of treatment conducted. Furthermore, drying and high temperatures have a significant effect on the changes of moisture content in seeds, which would then negatively affect seed viability [64]. This may be supported by biochemical analyses of isolated seed proteins that were conducted on soybean (Glycine max) seeds which are regulated under 22 °C (control), 24 °C (moderate), and 26 °C (extreme) day/night temperatures [65]. It was reported that the accumulation of lipoxygenase, the β-subunit of β-conglycinin, the sucrose-binding protein, and the Bowman–Birk protease inhibitor have been deteriorated by the extreme heat stress.

The low temperature can also affect the composition of other compounds or metabolites, along with carbohydrates, proteins, and fatty acids in the seeds. Findings from Xue et al. [66] revealed that, between the different incubation temperatures (15 °C, 25 °C, and 30 °C on the germination of pecan seeds, or Carya illinoensis), metabolites showed the most significant differences when seeds were incubated seeds under 30 °C. Hub metabolites found were mostly related to amino acids, which include, valine, threonine, serine, lysine, citrulline, 3-hydroxy- proline, phenylalanine, methionine, and ornithine. In addition,

organic acids such as oleic acid, linoleic acid, malonic acid, and palmitic acid were also significantly found in seeds that were incubated under 30 °C.

1.2.2. Reactive Oxygen Species (ROS) Production and Electrolyte Flux

The production and accumulation of reactive oxygen species (ROS) are one of the mechanisms in cellular metabolic activity of seeds when triggered by abiotic factors, such as high temperature and drought stress, that cause the changes in water content [67,68]. The uncontrolled accumulation of ROS can induce cellular damage, which can consequently be lethal to the seeds [69]. After shedding, the production and accumulation of ROS in recalcitrant seeds are still under control. However, during seed desiccation, the metabolic balance is interrupted as the production and accumulation of ROS become excessive (Figure 2). This has caused seed deterioration where the protective antioxidative reactions by antioxidants, such as glutathione (GSH), ascorbate peroxidase (APX), and ascorbic acid, are unable to effectively remove ROS from the system [70].

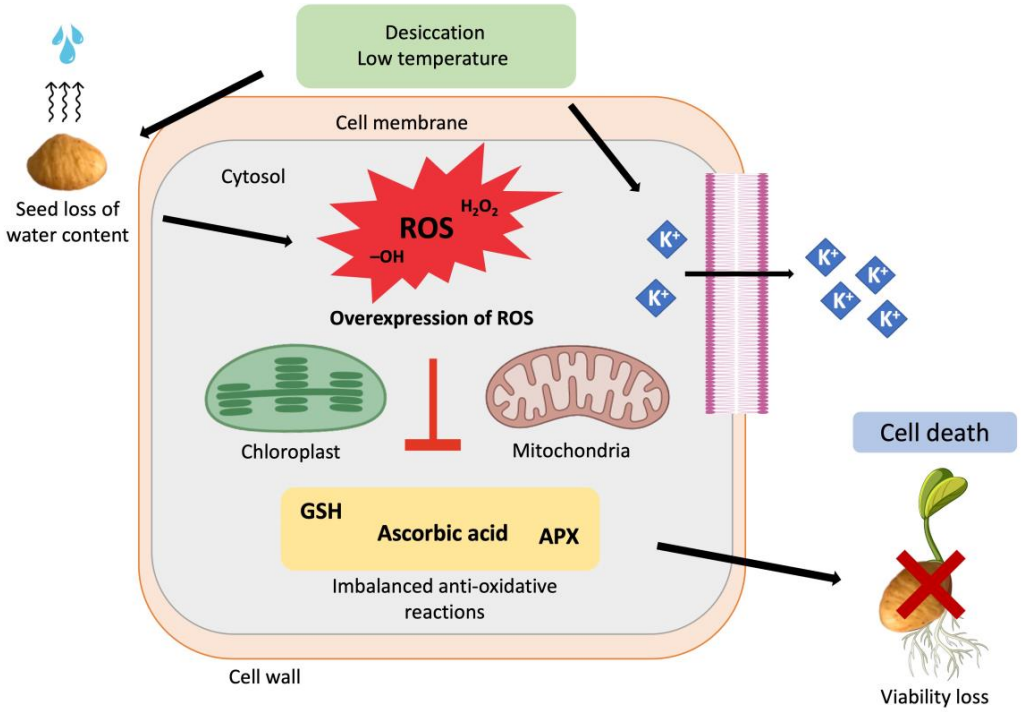

Figure 2. Recalcitrant seed losing its viability after oxidation stress from overexpression of ROS (reactive oxygen species) and imbalanced antioxidant production and electrolyte leakage due to desiccation and low temperature. APX: ascorbate peroxidase, GSH: glutathione, H_2O_2: hydrogen peroxide, K^+: potassium ion, −OH hydroxyl group.

Alongside the production of ROS during stress response, electrolyte leakage also occurs. Electrolyte leakage is an indicator of stress response in intact plant cells [71]. As the duration of the exposure of seeds to drying increases, the electrolyte leakage is found to increase as well [72]. Principally, electrolyte leakage is related to potassium ion and K^+ efflux from plant cells, which is regulated by plasma membrane cation conductivity. This event is used as a parameter to measure the occurrence of plant tissue injury, which occurs when stress is induced. It is also used to evaluate a plant's tolerance to stress [73]. Electrolyte leakage can reflect the effects of both metabolic (ROS-mediated lipid peroxidation) and mechanical or physical membrane damage on seeds brought on by desiccation [74]. However, electrolyte leakage may not always predict viability loss because membrane damage occurs before viability loss during the desiccation of recalcitrant seeds [75].

1.2.3. Gene Alteration in Seeds during Desiccation

During the desiccation process, some gene expressions in seeds have been altered, as well as various genes that play critical roles in regulating the response to water loss and the preservation of seed viability. Several gene families and pathways are known to be involved in desiccation tolerance in seeds, including late embryogenesis abundant (LEA) genes that are specifically linked to desiccation tolerance and have a vital function in protecting cells and cellular constituents during the process of seed desiccation. Some examples of LEA genes involved in desiccation or drying are *CsLEA* genes *LEA-1*, *LEA-2*, *LEA-3*, *LEA-4*, *LEA-5*, and *LEA-6* [26,76]. These genes are expressed during seed desiccation and believed to protect cellular structures from damage caused by water loss. Moreover, dehydrins (DHNs), Group 2 of LEA (late embryogenesis abundant) proteins, are induced under water deficit [77]. They are constituent elements of the developmental process in orthodox seeds but also have been identified in recalcitrant seeds such as *Acer saccharinum*, *Aesculus hippocastanum*, *Araucaria angustifolia*, *Camellia sinensis*, *Castanea sativa*, and *Poncirus trifoliate* [78]. Examples of dehydrin genes identified under water deficit are *QrDhn1*, *QrDhn2*, *QrDhn3*, and *DN949901*. In addition, other dehydrin genes are listed in the Table 2.

Along with LEA and dehydrin genes, ABA-responsive genes also play an important role in water-deficit response. Stomatal closure in guard cells is aided by ABA controlling solute efflux, which regulates the expression of numerous genes, some of which may play a role in enhancing tolerance to dehydration in both plant leaves and seeds [79]. According to Nakashima et al., dehydration-responsive elements (DREs) act as connecting elements between ABRE and the expression of *RD29A* when responding to ABA [80]. From the findings, an expression analysis using *abi3* and *abi5* mutants showed that *ABI3* and *ABI5* play important roles in the expression of *RD29B* in seeds. Furthermore, as seeds undergo desiccation, they can experience oxidative stress due to the accumulation of reactive oxygen species (ROS) [81]. Antioxidant genes, such as *SOD1*, *APX1*, *CAT1*, and *PDH*, help mitigate oxidative damage caused by ROS and maintain seed viability during drying [82]. The functions of catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and proline might have synergistically interacted to scavenge ROS [81].

There are genes that are responsible for storage proteins, which play vital roles in seed desiccation as they are involved in gathering and storing proteins that serve as a source of nutrients for the developing embryo during germination [83]. These storage proteins are crucial for seed development and early seedling growth. One of the examples is zein genes in maize, a class of storage proteins found in maize (corn) seeds that play a vital role in seed development and germination [84]. In addition, their content of proline enhances the protein's stability and safeguards it from desiccation stress. Another example of genes that encode storage protein are oleosin genes in oilseeds, such as rapeseed, sunflower seeds, and sesame seeds [85]. These genes are associated with lipid bodies and play a role in storing and safeguarding oil reserves during seed desiccation. Meanwhile, legumin and vicilin genes are genes that encode for two major classes of storage proteins found in legume seeds, such as soybeans and beans [86]. These genes act as a storage of nitrogen and amino acids for the developing embryo during the germination of the seed [83].

Seed-dormancy changes are predominantly induced by temperature, as well as water stress [87]. During water stress, aquaporins, which are membrane-transport proteins, regulate water movement across cell membranes [88]. Some specific aquaporins are expressed in seeds during desiccation and contribute to controlling water loss and rehydration processes [89]. Examples of aquaporin genes that are expressed in seeds during desiccation are *TIP3;1*, *TIP3;2*, *GmPIP2;9*, *OsPIP1;1*, *ZmPIP1;*, *AtNIP4;1*, *AtNIP4;2*, *CsSIP2;1*, and *CsXIP* [89,90]. During seed germination, certain TIPs, such as TIP1s, have been observed to play a role in vacuole biosynthesis, facilitating water movement into vacuoles [89]. This process leads to the mobilization of reserve substances, the maintenance of cell turgor pressure, and the promotion of embryo cell elongation. Moreover, several PIPs, including PIP1s and PIP2s, are involved in water exchanges between extracellular and cytoplasmic compartments, which are essential for maintaining water balance within the cytoplasm.

Table 2. Genes that are involved during seed desiccation and their examples.

Genes	Examples	References
LEA genes	CsLEA genes *LEA-1*, *LEA-2*, *LEA-3*, *LEA-4*, *LEA-5*, *LEA-6*	[26] [76]
Dehydrin genes	*QrDhn1*, *QrDhn2*, *QrDhn3*, *DN949901*, *Qp_Dhn1*, *Qp_Dhn2*, *Qp_Dhn3*, *Qp_Dhn4*, *Qp_Dhn5*, *Qp_Dhn6*, *Qp_AM711636*, *Qp_AM711635*	[78]
ABA-responsive genes	*ABI3*, *ABI5*, *AREBI*, *AREB2*, *RD29A*, *RD29B*	[80]
Antioxidant genes	*SOD1*, *APX1*, *CAT1*, *PDH1*	[82]
Storage protein genes	Zein genes, oleosin genes, legumin and vicilin genes	[84–86]
Aquaporin genes	*TIP3;1*, *TIP3;2*, *GmPIP2;9*, *OsPIP1;1*, *ZmPIP;1*, *AtNIP4;1*, *AtNIP4;2*, *CsSIP2;1* and *CsXIP*	[89,90]

2. Application of Systems Biology Approach on Recalcitrant Seed

A systems biology approach consists of omics analysis such as genomics, transcriptomics, proteomics, and metabolomics, along with bioinformatics. This review will provide insight into how each of these omics analyses assists in identifying the changes in the physiological and metabolic response of recalcitrant seeds after exposure to abiotic stimuli (e.g., desiccation and low temperature). Findings from the omics analysis of recalcitrant seed studies will be beneficial to understand the recalcitrance behaviour and for determining the key factors involved in storing recalcitrant seeds. With the advancement of recent technologies, such as genome sequencing, RNA-Seq, and LC-MS/MS technologies, the mechanisms that occur when recalcitrant seeds are exposed to unfavourable conditions can be studied from the level of genes to the metabolites. Since the majority of tropical fruits and crops are recalcitrant, this study could help in the effort of seed storage, mass propagation, and conservation of this desiccation-sensitive species.

2.1. Genomics and Molecular Markers

The goal of genomics research is to better understand the genome's structure and functions in a particular species. The study of genomics includes gene mapping, DNA sequencing, and the interaction of genetic and environmental variables in living organisms [91]. Meanwhile, transcriptomics study the whole collection of RNA transcripts generated by the genome at any particular moment, emphasizing how transcript patterns are altered [92]. Interestingly, both genomics and transcriptomics analyses are interconnected and have been used to identify genes that are expressed in whole seeds at different stages of development [93].

From the previous studies on recalcitrant mangosteen species, genome sequencing was conducted to investigate its genome composition and a first attempt at genome assembly by using the Illumina HiSeq 2000 sequencing platform [19]. There is a shortage of molecular genetics knowledge for this recalcitrant mangosteen plant, which hinders genetic studies and crop development for this commercially significant fruit tree. Findings from genomics studies can help to identify specific genes that are involved in the recalcitrant nature of certain species. By comparing gene expression patterns between recalcitrant and non-recalcitrant species, researchers can pinpoint genes that are differentially expressed and potentially associated with recalcitrance. This information can shed light on the molecular pathways and regulatory networks involved in recalcitrant behaviour.

Therefore, findings from these studies and sequence data are significant for genome surveys as they will provide information on G–C content, heterozygozity, and estimated genome size. Additionally, another study using whole genome sequencing on another mangosteen species has been conducted using single-molecule real-time (SMRT) sequencing data to impart crucial long sequences spanning repeats for the scaffolding of draft genome assembly from short reads, which later will be beneficial for improving genetic information on recalcitrant mangosteen species for gene annotation and further studies [20].

2.2. Transcriptomics Studies on Recalcitrant Seeds

Transcriptomic analysis on recalcitrant seeds of *Panax notoginseng* was studied by Yang et al. [30]. They identified 78 differential expressed genes (DEGs) that were related to seed-dormancy release at different after-ripening stages of the species. From the 78 DEGs, 15 were believed to associate with abscisic acid (ABA) and gibberellin. Abscisic acid is known to induce seed dormancy during embryo maturation. Meanwhile, gibberellin plays a role in promoting seed germination. Moreover, 26 DEGs that encoded late embryogenesis abundant (LEA) proteins and antioxidant enzymes were involved with desiccation tolerance in seeds. In Chinese cork oak (*Quercus variabilis*), a comparative transcriptome analysis on the desiccation sensitivity of the seeds revealed differentially expressed genes (DEGs) that were related to plant hormones (abscisic acid (ABA) and indole-3-acetic acid (IAA) biosynthesis), signal transduction, stress-response proteins (late embryogenesis protein (LEA) and heat-shock proteins (HSP)), and phospholipase D [28]. The findings exhibited a better understanding of the molecular regulation mechanism of desiccation sensitivity of *Q. variabilis* seeds.

Another transcriptomic analysis on a recalcitrant seed is conducted on tea-revealing expression profiles of 12 selected genes related to seed-dehydration treatment [25]. The selected genes included abscisic acid (ABA) biosynthesis, signal transduction, antioxidant enzymes, and late embryogenesis-abundant (LEA) protein genes. However, another study on the tea cultivar Echa 1 reported 87 *CsLEA* genes, while 48 genes were involved in seed development and 39 genes were involved in seed maturity and dehydration [26]. According to the findings, 16 *CsLEA* genes were thought to be involved in seed desiccation but were eventually suppressed. *CsLEA* genes respond to low temperatures, indicating their involvement in abiotic stress tolerance. Further analysis was conducted using Hidden Markov Model (HMM) profiles to search for genes that encode LEA proteins by detecting two tea plant genomes and three transcriptomes for all potential *CsLEA* genes. *CsLEA* genes' expression profile analyses were detected by using RNA isolation and qRT–PCR analysis at four different desiccation times (0, 3, 5, and 8 days) [26].

RNA sequencing with RT-qPCR on mangosteen seed germination from Day 0 to Day 7 was conducted to analyze differentially expressed genes (DEGs), which helps to understand the molecular mechanism of this recalcitrant seed during germination [21]. This study has revealed that abscisic acid (ABA) signalling has a role in stress response, whereas gibberellin (GA) promotes growth potential during mangosteen seed germination. On Day 3, it was determined that during the germination of mangosteen seeds, an increase in active transcripts leads to an elevation of ABA in the upregulated DEGs. However, among all phytohormones, GA exhibited the highest percentage of active transcripts, experiencing a significant decline on Day 3.

2.3. Proteomics Studies on the Recalcitrant Species

Proteomics is the study of the proteome, which includes structural characterization of proteins and their higher-order complexes, as well as the quantifications of the total number of proteins, protein isoforms, and modifications in a cell [94]. Proteomics complement other omics technologies by investigating the structure and functions of a particular protein [95].

In a study of the recalcitrant seed of tea (*Camellia sinensis*), desiccation treatment caused the build-up of hydrogen peroxide in the seed, as well as an increase in the activities of antioxidant enzymes, such as ascorbate peroxidase (APX) and superoxide dismutase (SOD) [27]. Proteomics analysis was conducted using 2D gel electrophoresis, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS), or tandem mass spectrometry (MS/MS) analysis, as well as non-redundant searching of the National Resource for Biotechnology Information (NCBI) putative protein database, which revealed 23 proteins associated with defense response, metabolism, and redox status upon desiccation exposure [27].

Protein identification conducted on the seed development of the recalcitrant *Ocotea catharinensis* Mez. using nanoLC-MS/MS analysis resulted in the identification of pro-

teins that were predominantly related to oxidative metabolism and synthesis of storage [31]. Among the 13 identified proteins, granule-bound starch synthase I, UDP-glucose-pyrophosphorylase (UDPase), and frutose–biphosphate aldolase were linked to the reserve's synthesis. The expression increased at the mature stage. The granule-bound starch synthase I contributed to the extra-long chains in amylopectins, the most common type of α-glucans in starch [96,97]. Meanwhile, the other two proteins were enzymes essential for cell-wall production and glucose metabolism [98].

In another study on proteome analysis on recalcitrant seed species, Moreton Bay chestnut (*Castanospermum australe*) indicated 10 polypeptides from 16 LEA transcripts were detected via liquid chromatography–electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). These polypeptides included highly abundant dehydrins such as CaDHN3 and CaCAPLEA-1 [32]. The heat-stable proteome was characterized in both recalcitrant *Castanospermum australe* seeds and the orthodox seed of *Medicago truncatula*, considering that LEA proteins serve an important role as protective molecules both in drought and in desiccation tolerance. Subsequently, the findings showed that the LEA polypeptides were either absent or significantly decreased in the recalcitrant *Castanospermum australe* seed as compared with the orthodox seed of *Medicago truncatula*. In addition, the absence or decline of LEA proteins in the seed of *Castanospermum australe* revealed a deeper understanding the molecular mechanism of recalcitrant seed traits.

2.4. Metabolomics Studies on the Recalcitrant Species

Metabolomics is a systems biology approach that provides an extensive understanding of an organism's biochemical via metabolite analysis [99]. Metabolomics involves metabolite profiling using mass spectrometry (i.e., Gas Chromatography–Mass Spectrometry (GC–MS) and Liquid Chromatography–Mass Spectrometry (LC–MS) for identifying and quantifying metabolites (small molecules), followed by multivariate methods for information extraction and data interpretation [23]. By using metabolomic analysis, the profiling of these metabolites can be achieved for a greater understanding of their roles in the metabolism of seed development.

For example, the metabolomic analysis of recalcitrant seeds of mangosteen at different stages using liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) showed the detection and quantification of various metabolites, such as flavonoids, organic acids, sugars, and amino acids [22]. The results indicated that the metabolites belonging to the citric acid cycle, sugar metabolism, and the phenylpropanoid pathway varied across the mangosteen seed developmental stages. The results also showed that the changes of metabolites, such as sugars, amino acids, organic acids, and glycosides, in response to different developmental stages of mangosteen seeds can be used as an indicator to determine the response of mangosteen seeds to environmental conditions because of their recalcitrance behaviour.

Meanwhile, different types of primary and secondary metabolites, such as sugars, organic acids, amino acids, alcohol, aldehydes, glycosides, fatty acids, phenolics, alkaloids, terpenoids, xanthones, and quinone were detected in mangosteen seeds [100]. These metabolites are related to the physiological and biochemical processes during mangosteen ripening in all mangosteen tissues, including seeds [23]. Secondary metabolites play an important role in the plant-signalling system as a response to abiotic stress conditions, such as environmental stresses like high-light intensity, drought stress, sugar, and nutrient deficiency [101]. A study by Mazlan et al. [52] detected metabolites such as D-glucose, glycine, glycerol, dibenzonquinoline, citrate, and tartaric acid. Moreover, the detection of chlorogenic acid, rutin, gambariin A1, and multiflorin B in mangosteen seeds is also reported [22]. From these studies, it was discovered the changes in sugar accumulation occurred during mangosteen seed development. Sugar accumulation in mangosteen seeds is accordant with the data from the previous report in other species of recalcitrant seeds. In addition, it was reported there were changes in the organic acid and flavonoid levels in the seed development. Moreover, metabolite changes were detected in the mangosteen tissues,

including the seeds at different ripening stages (Stage 0, 2, 4, and 6), [23]. For instance, arabinofuranose was detected in the early stages of ripening, which are Stages 0 and 2. The abundance of this metabolite decreased at the final ripening stage, particularly in the pericarp and seed. However, other sugars, such as L (-)-fucose, were detected at Stage 2 in the seed, whereas L-mannopyranose was detected at Stage 2 in the pericarp. It was determined that the abundance of L (-)-fucose had increased in the seed of mangosteen from Stage 2 until Stage 6 [23]. In addition, based on [24], 69 of the metabolites were identified from the elucidation of metabolic changes during the mangosteen ripening stages of the seed. Furthermore, the correlation between mangosteen ripening stages in mangosteen tissues, including seeds with the production of metabolites such as phenylalanine, valine, isoleucine, serine, and tyrosine [24], was determined. These metabolite regulations could be linked to physiological and biochemical processes during ripening, such as cell-wall degradation and plant defenses in recalcitrant species.

Studies on *Quercus robur* seeds during storage at subzero temperatures (−3 °C and −7 °C) revealed that seeds showed deterioration, while seed germination declined at −7 °C [29]. Moreover, an increased amount of phenolic chemicals, carbohydrates, amino acids, and phosphorylated monosaccharides were notably detected in this study. It was found that in seeds stored at 7 °C, there was an increase in the abundance of defense-related metabolites (1,2,4-Benzenetriol; BTO), ascorbic acid-degradation products (threonic and isothreonic acid), and anti-freezing compounds (sugar alcohols, primarily threitol). These findings can affirm the recalcitrance behaviour of the *Quercus robur*.

3. How Multi-Omics Integration Can Assist in Understanding the Recalcitrance Behaviour

The multi-omics integration method for assimilation, annotation, and modelling large datasets from all omics studies can be divided into three levels: element-based integration (Level 1), pathway-based integration (Level 2), and mathematical-based integration (Level 3) [102]. Each level approach can assist the comprehensive study within each level, particularly how they are associated and contribute to dataset outcomes. In addition, by integrating omics data with mathematical models, This technique could have a high potential for the greater understanding of plant metabolisms [103]. Hence, the integration of omics data with the above-mentioned mathematical models can aid in the discovery of recalcitrance behaviour. Table 3 summarizes all recalcitrant species studies using the omics approach.

Table 3. Summary of recalcitrant species studies using omics approaches and their findings.

Species	Condition/Treatment	Tools or Platforms	Main Findings	References
		Genomics		
Mangosteen (*Garcinia mangostana*)	Red young leaf tissues from 4-to-5-month-old plants.	Illumina HiSeq 2000	Genome sequence data of *Garcinia mangostana*.	[19]
Mangosteen (*G. mangostana* var. Mesta)	Red young leaf tissues from 4-month-old plants.	SMRT–Seq	Genome sequence data of *G. mangostana* var. Mesta.	[20]
		Transcriptomics		
Ginseng (*Panax notoginseng*)	Harvested seeds 0, 20, 40, and 60 days after ripening (DAR).	RNA–Seq by Illumina Hiseq 2500	Seventy-eight DEGs related to seed-dormancy release at different after-ripening stages. Fifteen DEGs associated with abscisic acid and gibberellin. Twenty-six DEGs that encode LEA protein and antioxidant enzymes were correlated with desiccation tolerance in seeds.	[30]

Table 3. *Cont.*

Species	Condition/Treatment	Tools or Platforms	Main Findings	References
Chinese cork oak (*Quercus variabilis*)	Dehydration of seeds with silica gel for Days 0, 1, and 15.	qRT-PCR, Ilumina Hi-Seq 4000	Differential expressed genes (DEGs) that are related to plant hormones (abscisic acid (ABA) and indole-3-acetic acid (IAA) biosynthesis, signal transduction, stress-response proteins (late-embryogenesis protein (LEA), and heat-shock protein (HSP)), and phospholipase D.	[28]
Tea (*Camellia sinensis* L.)	Dehydration of seeds for Days 0, 1, 3, 5, 8, 11, 14, and 18.	RNA–Seq by Illumina Hiseq 2500 and qRT-PCR	Expression profiles of 12 selected genes related to seed-dehydration treatment.	[25]
Tea (*C. sinensis* cv. Echa 1)	Seeds at four different desiccation time stages (Days 0, 3, 5, and 8).	qRT–PCR, NanoDrop 2000 spectrophotometer	Thirty-nine *CsLEA genes* may be involved in seed maturity and dehydration.	[26]
Mangosteen (*Garcinia mangostana* L.)	Seeds at four different times after sowing Days 0, 3, 5, and 7.	NanoDrop ND–1000 spectrophotometer, HiSeq 2000 Ilumina	Increased active transcripts during mangosteen seed germination.	[21]
		Proteomics		
Tea (*Camellia sinensis* Kuntze)	Seed desiccation with 15% relative humidity and 15 °C at different intervals of time.	2D gel electrophoresis, MALDI–TOF MS, or MS/MS analysis	Build-up of hydrogen peroxide, an increase in the activities of antioxidant enzymes (ascorbate peroxidase (APX) and superoxide dismutase (SOD). Twenty-three proteins were associated with defence responses, metabolism, and redox status upon desiccation exposure.	[27]
Catharina's Ocotea (*Ocotea catharinensis* Mez.)	Different stages of seed-embryo development (170, 230, and 300 days).	nanoLC–MS/MS	Thirteen proteins associated with the reserve synthesis were observed to increase at the mature stage.	[31]
Moreton Bay chestnut (*Castanospermum australe*)	Seed drying over a saturated salt solution at 75% RH (0, 2, 4, 6, and 8 h).	LC–ESI–MS/MS	Ten LEA polypeptides consist of the dehydrins that were profiled.	[32]
		Metabolomics		
Mangosteen (*Garcinia mangostana*)	Different developmental stages of seeds.	Liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS)	Seventeen metabolites, such as flavonoids, organic acids, sugars, and amino acids, were profiled.	[22]
Mangosteen (*Garcinia mangostana*)	Different ripening seed stages (Stages 0, 2, 4, and 6).	Liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS)	Twenty-four metabolites, such as flavonoids, organic acids, sugars, and amino acids, were profiled.	[23]
Mangosteen (*Garcinia mangostana*)	Different ripening seed stages (Stages 0, 2, 4, and 6).	Gas chromatography–mass spectrometry (GC–MS)	Sixty-nine metabolites were identified, including phenylalanine, valine, isoleucine, serine, and tyrosine.	[24]

Table 3. *Cont.*

Species	Condition/Treatment	Tools or Platforms	Main Findings	References
Pedunculate oak (*Quercus robur* L.)	Cold temperature treatment of seeds (−3 °C and −7 °C).	Gas chromatography-tandem mass spectrometry (GC–MS/MS)	Forty-four differentially abundant metabolites, such as 1,2,4–Benzenetriol, catechin, and soluble carbohydates, were detected.	[29]

The utilization of advanced technologies and tools will aid the multi-omics data integration process. For instance, Pinu et al. [104] highlighted a database resource, the Kyoto Encyclopedia of Genes and Genomes (KEGG), that has a collection of genomes, biological pathways, and chemical compound databases, all of which will ease data searching. Moreover, powerful multivariate software (e.g., SIMCA, Unscrambler-X) can easily build principal component analysis (PCA) and partial least-square-discriminant analysis (PLS-DA) models to highlight important trends, clusters, and patterns in the data without the need for programming skills. Moreover, Fukushima et al. [103] highlighted the use of genome-scale metabolic reconstruction using modelling and mathematical simulations.

A multi-omics integration approach may assist in investigating the physiological and metabolic behaviour of recalcitrant seeds. For example, the combination of genomics, transcriptomics, and proteomics analysis that involved suppression subtractive hybridization (SSH) and gel-based and gel-free profiling was conducted by Romero Rodríguez et al. [105]. The results revealed the protein-coding genes involved in desiccation tolerance, ABA signaling control, metabolism, and antioxidative defense, as well as the genes expressed and transcripts involved in disclosing the metabolic state of the recalcitrant seed of *Quercus ilex*. Another multi-omics analysis of recalcitrant *Quercus ilex* was conducted by López–Hildago et al. [106] using high-throughput technologies, including NGS–Illumina for transcriptomics, shotgun LC–MS/MS for proteomics, and Gas–Chromatography-Mass Spectrometry (GC–MS) for metabolomic studies. From the analyses, approximately 62,629 transcripts, 2380 protein species, and 62 metabolites were identified, which allowed for visualization and metabolic pathway reconstruction. The integration of all omics analysis using respective technologies in order to understand the recalcitrant behaviour mechanisms, [107], which can be accommodating to prolong the seed storage of this recalcitrant species, is shown in Figure 3.

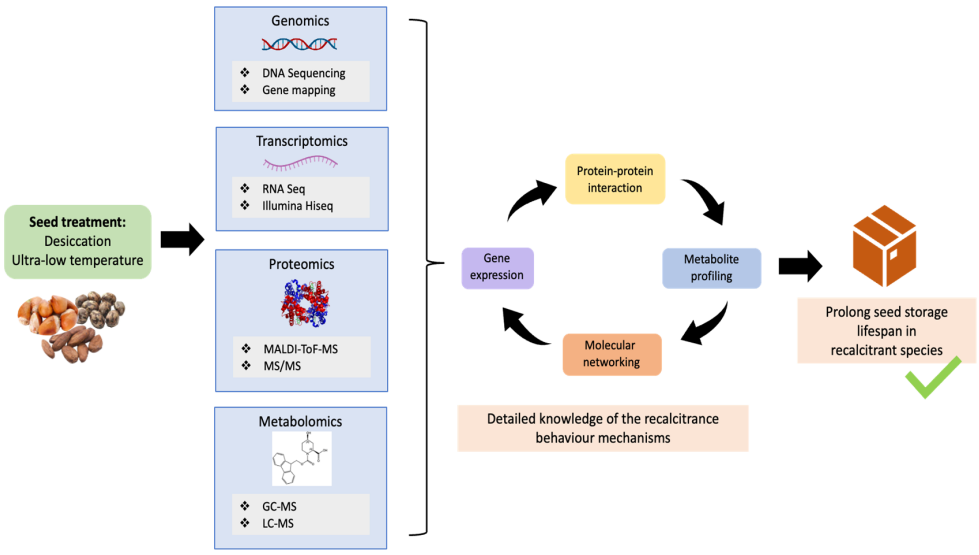

Figure 3. An overview of multi-omics integration and its application in understanding recalcitrance behaviour, as well as contribution to seed storage of recalcitrant species.

4. Conclusions

Systems biology has the potential to improve our understanding of biological changes in recalcitrance, as these desiccation-sensitive and low-temperature-sensitive characteristics have hindered the conservation and storage of plant species with this recalcitrance behaviour. Thus, there is a need to study genes, transcripts, proteins, and metabolites involved or expressed when exposed to desiccation and low temperatures. The significance of this study is not only to find suitable alternative to store recalcitrant seeds for a long time, but also to determine the best method with seeds that possess the recalcitrance behaviour to make the seed storage more manageable. The combination of omics analyses and the integration of multi-omics ease the effort of discovery of compounds or components involved in recalcitrance behaviour. This knowledge can help for the understanding of recalcitrant seed behaviour and improve crop management in terms of seed storage for conservation and sustaining plant biodiversity.

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