Desiccation tolerance in seeds and plants

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

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Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 8 January 2016
at 1:30 p.m. in the Aula.

Maria Cecília Dias Costa
Desiccation tolerance in seeds and plants,
184 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-627-8

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General Introduction

Seeds of the majority of modern angiosperm species (90%) tolerate dehydration to very low water contents (orthodox seeds) (Royal Botanic Gardens Kew, 2008). This means that orthodox seeds possess the most amazing ability in plant biology, namely the ability to tolerate desiccation (Bartels and Salamini, 2001). Desiccation tolerance (DT) is amazing because it implies that an organism is able to suspend growth and development until water is available again (survive in the dry state) (Alpert, 2006). For seeds, survival in the dry state allows the formation of soil seed banks, to travel further away from the mother plant and expand the species habitat range (Franchi et al., 2011). Together with seed longevity (lifespan of seeds in dry storage), DT provides a highly effective strategy for successful transmission of genetic information from the mother plant to the next generation and is the basis for *ex situ* conservation of orthodox seeds (Waterworth et al., 2015). Moreover, seed DT combined with dormancy ensures that germination will only occur at the appropriate time to optimize seedling establishment, influences the rate at which species expand their range and determines the chances of survival or extinction upon climate change (Linkies et al., 2010).

During maturation, orthodox seeds achieve water contents as low as 5-10% on a fresh weight basis and can even be further dried to 1-5% without accumulating major damage (Kermode, 1997). In the dry state, these "mysterious genetic capsules" are resistant to a broad range of harsh conditions that are lethal to most adult plants (Nambara and Nonogaki, 2012). For example, mature seeds of *Ipomoea grandiflora, Merremia cissoids* and *Merremia aegyptia* not only survive fire but exposure to such high temperatures even stimulates germination (Azania et al., 2003). *Arabidopsis thaliana* and *Nicotiana tabacum* seeds germinated after 1.5 years of exposure to solar UV, solar and galactic cosmic radiation, temperature fluctuations and space vacuum outside the International Space Station (Tepfer et al., 2012). Plants from zones with harsh winters produce over-wintering seeds that are resistant to freezing (Shimada et al., 2008). Besides not being lethal for orthodox seeds, freezing temperatures are beneficial for their long-term conservation in seed banks. In the dry state, seeds are also very resistant to the passage of time. Spectacular cases of seed longevity have been reported. Seeds from three Egyptian *Acacia* species maintained in Swedish museums

germinated after 151 years of storage (Leino and Edqvist, 2010). Seeds of *Leucospermum* sp., *Liparia* sp. and *Acacia* sp. germinated after more than 200 years (carbon dated at 218-270 years) under suboptimal storage condition (Daws et al., 2007b). Seeds of Sacred Lotus (*Nulembo nucifera*) from an ancient lake bed at Pulantien, Liaoning Province, China, showed exceptional long-term viability by germinating while they were 1,288 years old (carbon dated at 1,350±220 years) (Shen-Miller et al., 1995). All these examples point at the existence of a genetically programmed stress endurance capacity used by seeds to sense environmental factors and determine whether they will germinate or not (Kranner et al., 2010; Nambara and Nonogaki, 2012). This capacity is due to a set of protection and repair mechanisms that together determine the traits of longevity, dormancy and DT (Kranner et al., 2010).

Orthodox seeds represent the ultimate example of DT, but they are not the only life form that tolerates desiccation. We find examples of DT also in yeasts, crustaceans, nematodes, rotifers, tardigrades and whole plants. In plants, DT was primitively present in mosses, liverworts, lichens and algae (Alpert, 2000). It is very likely that DT arose with the transition from aquatic to terrestrial life forms, in organisms living in seashores and tidal habitats, where both the probability of experiencing adverse conditions and the survival cost of such conditions were high (Jönsson and Järemo, 2003). In these organisms, cycles of dehydration and rehydration were crucial to cell formation and functioning. Later, as plants expanded their land habitat, which resulted in the establishment of more complex ecosystems, a drawback became evident: desiccation tolerant plants grow slowly (Alpert, 2006). Shutting down metabolism early during drying and fully recovering upon rehydration consumes energy, which can be used for growth by desiccation sensitive species (Alpert, 2006). This trade-off between DT and growth favoured the loss of DT in vascular plants (Alpert, 2006; Oliver et al., 2000). DT-related genes were then recruited for processes such as the response to water stress, improved control of water status, vegetative cellular protection and repair, and DT of spores, pollen grains and seeds (Illing et al., 2005; Oliver et al., 2000, 2005). Nowadays, DT is common in pteridophytes, rare in angiosperms and absent in gymnosperms (Porembski, 2011).

In total, it is estimated that only about 1,300 species of vascular

plants, of which 135 are flowering plants (termed “resurrection plants”) are able to display DT in their vegetative tissues (Black and Pritchard, 2002; Gaff, 1971; Illing et al., 2005; Porembski, 2011). These plants occur predominantly in shallow soils on rocky outcrops in semitropical and tropical regions of Africa, America and Australia (Alpert and Oliver, 2002; Gaff, 1971, 1977, 1987). Under the harsh microclimatic and edaphic conditions of these areas, resurrection plants are not outcompeted by faster growing desiccation-sensitive plants (Porembski, 2011).

Phylogenetic evidence suggests that resurrection plants regained the ability to tolerate desiccation of their vegetative tissues from mechanisms present first in bryophytes (Bartels and Hussain, 2011). In the history of angiosperms, this (re-)evolution occurred multiple times (at least thirteen), mostly within herbaceous lineages, but without an evident pattern of systematic distribution (Oliver et al., 2000, 2005; Porembski, 2011).

The DT of resurrection plants shares mechanisms with DT of seeds, as the same types of molecules are employed in both cases (Rascio and Rocca, 2005). For instance, sucrose and oligosaccharides were shown to confer DT to maturing seeds of soybean (*Glycine max*), pea (*Pisum sativum*) and maize (*Zea mays*) (Koster and Leopold, 1988), while in *Haberlea rhodopensis* leaves, the maintenance of constant high levels of sucrose and raffinose increased the chances of surviving desiccation (Djilianov et al., 2011). The accumulation of dehydrins was highlighted as a major attribute for DT in developing *Fagus sylvatica* seeds (Kalemba et al., 2008) as well as the leaves of the resurrection plant, *Craterostigma plantagineum* (Giarola et al., 2015). In barley (*Hordeum vulgare*) and the resurrection plant *Xerophyta viscosa*, the activation of genes related to antioxidants helped to maintain cell integrity against rehydration-induced damage (Ekmekci et al., 2005; Sreenivasulu et al., 2008). Therefore, it has been speculated that the vegetative DT in resurrection plants is an adaptation of seed developmentally-regulated DT mechanisms adjusted to the whole plant context (Farrant and Moore, 2011; Illing et al., 2005; Oliver et al., 2000).

One of the aims of DT studies is the generation of information useful for the development of crops with improved drought tolerance.

Some mechanisms identified by these studies have been successfully used to generate more stress tolerant plants. For example, the drought-responsive transcription factor VuDREB2A from cowpea (*Vigna unguiculata*) enhanced drought tolerance in transgenic *A. thaliana* by up-regulating a series of stress-responsive genes (Sadhukhan et al., 2014). The seed-specific HSFA9 transcription factor from sunflower (*Helianthus annuus*) conferred tolerance to severe dehydration to vegetative tissues of tobacco (*Nicotiana tabacum*) possibly by inducing the accumulation of small heat shock proteins (Prieto-Dapena et al., 2008). Overexpression of *MYB96* from *A. thaliana* increased drought resistance in transgenic camelina (*Camelina sativa*) plants by activating the expression of wax biosynthesis genes and accumulating wax load (Lee et al., 2014). Maize plants overexpressing the *Rab28* LEA gene exhibited sustained growth under osmotic stress and higher germination rates under water deficient conditions (Amara et al., 2013).

Further progress in improving drought-tolerance in sensitive plants can be made by the identification and characterization of DT-related genes in resurrection plants. For instance, the gene *XVSAP1* from *X. viscosa* enhanced tolerance to salinity, osmotic and high-temperature stress in transgenic *A. thaliana* plants possibly by stabilizing cell membranes (Garwe et al., 2006). Transgenic tobacco plants overexpressing the aldose reductase enzyme ALDRXV4 from *X. viscosa* showed improved photosynthetic efficiency, less electrolyte damage, greater water retention and higher proline accumulation under osmotic stress conditions compared to wild type plants (Kumar et al., 2013).

However, individual genes identified by DT studies might not have a substantial effect on improving stress tolerance in other species because they might be necessary but not sufficient for tolerance either because they require coordinated expression with other genes to be functional or they have limited effect on enhancing tolerance on their own (Iturriaga et al., 1992). The identification of key genes in regulatory networks controlling DT could be a more promising approach because they could enable the manipulation of multiple pathways that are required for tolerance of extreme dehydration (Farrant et al., 2015).

The plant hormone abscisic acid (ABA) has been considered a master regulator of plant development and water loss responses.

ABA regulates embryo and seed development, promotion of seed dormancy and DT, germination, seedling establishment, vegetative development, general growth and, during dehydration, accumulation of osmocompatible solutes and synthesis of dehydrins and LEA proteins (Cutler et al., 2010). ABA has been shown to accumulate in dehydrating tissues of a number of species, such as *A. thaliana* (Harb et al., 2010), *Chamaegigas intrepidus* (Schiller et al., 1997), *Citrus* sp. (Agustí et al., 2007), *C. plantagineum* (Bartels, 2005), *Sporobolus stapfianus* (Whittaker et al., 2001) and maize (Lü et al., 2007). In dehydrating tissues, ABA responses lead to changes in gene transcription, transcript processing and stability (Cutler et al., 2010). ABA-induced genes encode for proteins such as dehydrins, enzymes that detoxify reactive oxygen species, enzymes of compatible solute metabolism, a variety of transporters, transcription factors, protein kinases and phosphatases, and enzymes involved in phospholipid signalling (Cutler et al., 2010). Increasing the knowledge of ABA-dependent drought responses and its core signalling components may open up promising possibilities for development of drought tolerant crop plants.

Considering the predictions of a near future that is several degrees warmer, which will more often result in drought (Sherwood and Fu, 2014a), the pressure to produce crop varieties with improved drought tolerance is increasing. This pressure is the strongest in developing countries, which struggle to maintain robust breeding capabilities and need support for the development of stress tolerant crops critical for food security (Tester and Langridge, 2010).

Orthodox seeds and resurrection plants can serve as a model to understand the numerous interacting factors promoting DT (Farrant et al., 2015). Several studies have advanced our knowledge on such factors. One example is the ABA signalosome complex (PYR/PYL-PP2C-SnRK2) that regulates stress responses and seed development (Umezawa et al., 2010). When ABA levels increase due to environmental stimuli, the ABA molecule binds to PYR/PYL/RCAR receptors, that interact with PP2C phosphatases (Sreenivasulu et al., 2012). Then, these PP2Cs no longer inactivate downstream SnRK2 that then phosphorylates and thereby activates downstream targets such as AREB/ABF transcription factors or ion channels, leading to ABA responses such as stomatal closure

(Sreenivasulu et al., 2012). It has been shown that the interaction between ABA and ethylene regulates germination and is crucial for DT (Arc et al., 2013; Ndima et al., 2001). The last step in the biosynthesis of ethylene is susceptible to ABA inhibition, while ethylene down-regulates ABA accumulation by inhibiting its biosynthesis, promoting its inactivation and negatively regulating its signalling (Arc et al., 2013). In dormant seeds, ethylene counteracts the inhibitory effects of ABA, promoting germination (Arc et al., 2013). In plants, ethylene modulates drought stress signalling by acting antagonistically to ABA (Huang et al., 2008). ABA also induces the expression of genes encoding LEA proteins during seed development and abiotic stress (Bies-Ethève et al., 2008; Dalal et al., 2009; Kamisugi and Cuming, 2005; Zhao et al., 2011). In general, LEA proteins are hydrophilic, intrinsically unstructured and adopt specific three-dimensional structures on desiccation or in extreme temperatures (Bies-Ethève et al., 2008). LEA proteins have several possible functions, including roles as antioxidants, membrane and protein stabilisers during water stress and "space fillers" to prevent cellular collapse at low water activities (Tunnacliffe and Wise, 2007).

A last example of interacting factors promoting DT is the raffinose family oligosaccharides (RFOs). RFOs have been shown to participate in several crucial plant cellular functions including transport and storage of carbon, signal transduction, membrane trafficking and mRNA export and act as compatible solutes, signalling molecules and antioxidants in response to stress (Elsayed et al., 2014). In desiccated leaves of *Craterostigma* sp., *de novo* biosynthesis of RFOs induced by water deficit provides additional protection (Egert et al., 2015). In maturing seeds of *Medicago truncatula*, the conversion of sucrose into RFOs was linked to seed vigour during germination and seedling establishment (Vandecasteele et al., 2011).

Despite all these advances, many questions remain to be answered in respect to DT mechanisms. The work presented in this thesis intends to shed light on some of these questions.

Scope of the thesis

In this thesis, I use germinated orthodox seeds and resurrection plants as main experimental models. Orthodox seeds lose DT during germination and, up to a certain developmental stage, an osmotic and/or ABA treatment is sufficient to rescue DT in these seeds. In the second chapter, I present a review on the use of germinated seeds to study DT and discuss the ecological implications for seeds being able to re-acquire DT after germination. The role of ABA in re-induction of DT in germinated *A. thaliana* seeds is the theme of the third chapter. Microarray data from a time-series of DT re-induction are used to gain insight into the temporal changes in gene expression induced by ABA. In the fourth chapter, germinated seeds of rice (*Oryza sativa*) cultivars with contrasting levels of drought tolerance are used to analyse the relation between drought tolerance in vegetative tissues and DT in seeds. The influence of a treatment to re-induce DT on the longevity of germinated *Sesbania virgata* seeds is presented in the fifth chapter. The theme of the sixth chapter is the resurrection species *X. viscosa. X. viscosa* seeds lose DT during germination and re-acquire it progressively during seedling development. Incubating desiccation sensitive *X. viscosa* seedlings in ABA can induce tolerance to fast drying in shoots, but not in roots. In this chapter, I analyse the changes in the transcriptome and proteome of shoots and roots of *X. viscosa* seedlings before (desiccation sensitive) and after (desiccation tolerant) an ABA treatment. To improve the identification of transcripts and proteins, I also present a draft genome sequence of this species. The seventh chapter presents the results of a search for DT-conserved genes in different life forms. To do so, I compare transcriptomes of germinated seeds, resurrection plants, a lichen and a nematode in relation to DT. The outcomes highlight common mechanism for DT induction in seeds and vegetative tissues and a likely co-evolution of DT and dormancy. Finally, in the eighth chapter, I summarize and discuss the content of this thesis in the light of the potential of DT studies to contribute to world food security.

Chapter
Two

Acquisition and loss of desiccation tolerance in seeds: from experimental model to biological relevance

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Published in Planta, 241(3), 563-577

Abstract

Desiccation tolerance (DT) is the ability of certain organisms to survive extreme water losses without accumulation of lethal damage. This was a key feature in the conquering of dry land and is currently found in all taxa including bacteria, fungi, roundworms and plants. Not surprisingly, studies in various fields have been performed to unravel this intriguing phenomenon. In flowering plants, DT is rare in whole plants (vegetative tissues), yet it is common in seeds. In this review, we present our current understanding of the evolution of DT in plants. We focus on the acquisition of DT in seeds and the subsequent loss during and after germination by highlighting and comparing research in two model plants *Medicago truncatula* and *Arabidopsis thaliana*. Finally, we discuss the ability of seeds to re-establish DT during post germination, the possible ecological meaning of this phenomenon, and the hypothesis that DT, in combination with dormancy, optimizes seedling establishment.

Keywords

ABA, Arabidopsis, germination, Medicago, seed development, seedling establishment

Introduction

Life without water

Water is the most limiting resource in living systems. Water molecules constitute most of the cellular volume of plants, as well as of most other organisms. Due to their properties, water molecules are critical components of chemical reactions and contribute to the stability of proteins, DNA, lipids and membranes. How different organisms survive in the absence or under very limited amounts of water is still an open question. The first observations of such a phenomenon were made by Antonie van Leeuwenhoek, a Dutch tradesman and scientist, who recorded them in his letter 'On certain Animalcules found in the sediment in gutters of the roofs of houses'. In this letter, he describes how certain 'animalcules' (today's microorganisms) would contract themselves into an oval shape when dehydrated and unfold their bodies upon re-watering to regain life (Keilin, 1959). He repeated these experiments many times with the same success and even 'animalcules' that were in a dry sediment that was kept in his study for months, were competent to regain life. Insightfully, van Leeuwenhoek also hypothesized that if such organisms could stay so long in a dry state and regain life, this should be the way of survival in places where water bodies dry up during summer time or the dry season (e.g. in deserts). He also suggested that these 'animalcules' where likely transported from one place to another in the dried mud adhered to the feet or feathers of aquatic birds (Keilin, 1959). These two examples already illustrate important functions, in an ecological sense and in the life history of an organism, that can be attributed to the possibility of surviving in a dry state (anhydrobiosis) by tolerating desiccation. What van Leeuwenhoek did not mention, however, was that after the 'animalcules' were contracted in a ball shape they had minute amounts of water inside of them and were living in an anhydrobiotic quiescent state which we now know depends on the activation of a series of protective mechanisms including genes, proteins, and metabolites, collectively referred to as 'desiccome' (Leprince and Buitink, 2010; Potts et al., 2005).

Desiccation tolerance (DT) is defined as the ability of certain

organisms to deal with extreme water loss to levels below 0.1g H_2O per gram dry weight and subsequent re-hydration without accumulation of lethal damage (Alpert, 2005; Leprince and Buitink, 2010; Oliver et al., 2005). Thus, desiccation tolerant organisms usually do not avoid water losses; instead, they deal with water removal by equipping themselves with protective molecules and by entering into a quiescent, metabolically inactive state (Alpert, 2005). To date, a vast body of knowledge has been built around the understanding of anhydrobiosis, or DT (Alpert, 2005). Since the late 70s, studies of the physiology, physics, biophysics and, most recently, genetics of dry living systems have burst into bloom both on fundamental and applied aspects (reviewed by Alpert, 2006; Farrant and Moore, 2011; Gechev et al., 2012; Leprince and Buitink, 2010). Since the first observations by van Leeuwenhoek, the ability to withstand desiccation has been found in a wide array of organisms, including bacteria, yeast, fungi, roundworms, arthropods, and plants (Alpert, 2006).

DT research has diverse (potential) applications, such as improvement of drought tolerance in crop species, improvement of *ex situ* preservation of germplasm, stabilization of biomolecules and eukaryotic cells, and extension of the shelf-life of vaccines and biological materials, such as blood cells for transfusion and tissues for transplantation (Garwe et al., 2006; Kumar et al., 2013; Loi et al., 2013; Potts et al., 2005; Satpathy et al., 2004). Understanding the mechanisms of DT is an important step towards a multitude of plant and non-plant applications.

In plants, DT is rare in shoots and roots; however, it is common in seeds and pollen. Here, we present our current understanding of the evolution and mechanisms underlying DT in seeds. This review mainly deals with DT *sensu stricto*, i.e. the ability to survive extreme water loss, and the biological role of DT, which is also dependent on storability in the dry state (seed longevity). Desiccation tolerant seeds are not by definition long-lived, as seeds acquire longevity in a gradual fashion, late during development (Verdier et al., 2013). However, DT is a prerequisite for seeds to acquire longevity, implying strong interdependency between both traits. We describe the acquisition of DT in seeds and the subsequent loss during and after germination by highlighting and comparing studies in the two model plants *Medicago truncatula* and *Arabidopsis thaliana*.

Finally, we discuss the ability of seeds to re-establish DT during and after germination, the possible biological/ecological meaning of this phenomenon, and the hypothesis that DT in germinated seeds, in combination with dormancy, optimizes seedling establishment. Although we will briefly introduce strategies to deal with water limitation as well the challenges of withstanding desiccation, this is not the focus of this review and more details on this topic can be found elsewhere (e.g. Berjak and Pammenter, 2008; Dinakar and Bartels, 2013; Farrant and Moore, 2011; Gechev et al., 2012; Leprince and Buitink, 2010; Moore et al., 2008; Verslues and Juenger, 2011).

Different ways to deal with water limitation

Dehydration is a common stress and plants evolved in various ways to cope with it. Plants differ in their level of tolerance to dehydration and can be roughly divided in extremely tolerant, moderately tolerant and lowly tolerant (Fig 2.1). Extremely tolerant plants are desiccation tolerant and tolerate nearly complete dehydration. Plants with this extreme capacity are also known as resurrection plants (Gaff, 1971). These desiccation tolerant plants do not avoid water loss, but protect themselves against water removal by shutting down metabolism and activating protective mechanisms (reviewed by Farrant et al., 2007). Unlike resurrection plants, plants with a moderate tolerance remain hydrated, are metabolically active, and use the available water efficiently under water-restricted conditions (Verslues and Juenger, 2011). Many plants, however, have a low tolerance to water loss and depend for their survival on drought tolerance mechanisms (Verslues and Juenger, 2011). Drought tolerance (which is different from DT) denotes the capacity to tolerate moderate dehydration down to ~0.3 g H_2O per gram dry weight. Usually, drought refers to a temporary type of stress which is dealt with via the continuation of most of the physiological functions of the organism while preventing water loss, for example by limiting growth, stomata closure, and/or the accumulation of solutes (Moore et al., 2008). If the drought stress is too severe or the period of drought is too long, these drought tolerant organisms will eventually perish.

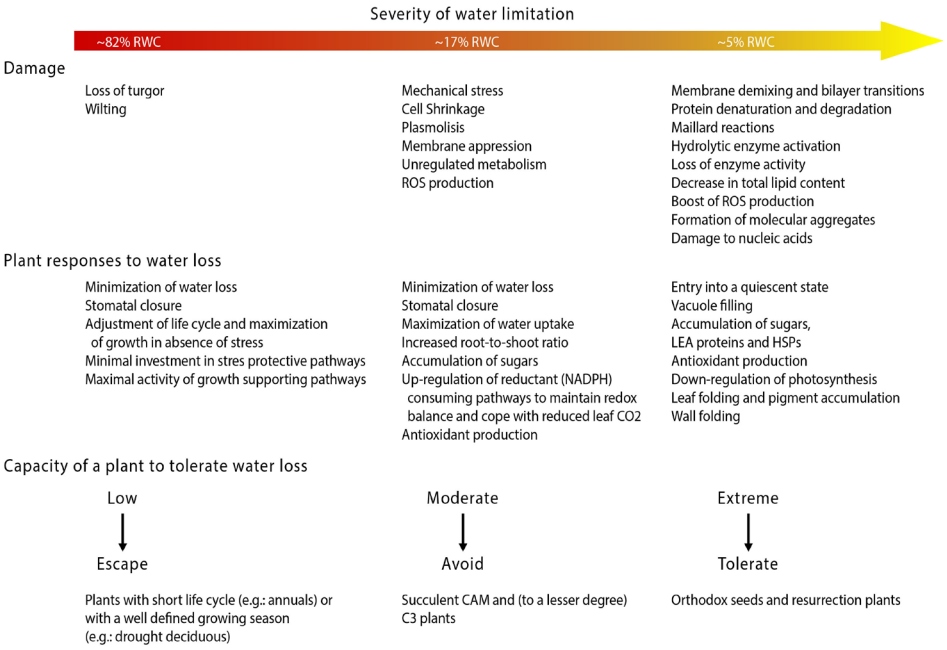


Fig 2.1 *Plant responses and adaptation strategies to different levels of water limitation. Plant adaptation strategies to water limitation can range from escape (e.g.: annuals and drought deciduous) to tolerance (orthodox seeds and resurrection plants) (Verslues and Juenger, 2011).*

Challenges and mechanisms to deal with desiccation

Cells that undergo desiccation have to cope with significant changes in turgor pressure, which generate cell shrinkage and mechanical stress (Farrant, 2000; Scoffoni et al., 2014). Other challenges faced by desiccating cells are the prevention of denaturation of large molecules, loss of enzyme activity, formation of molecular aggregates, and cellular damage caused by reactive oxygen species (ROS) (reviewed by Kranner and Birtić, 2005). Protective measures can either be (partly) present or induced by signalling and activation of transcription factors (Gechev et al., 2012). The effects of desiccation and the stresses it causes are as multifaceted as the response to desiccation. The response to desiccation comprises a complex array of protective mechanisms against mechanical stress caused by water removal, including modification of the cell wall and membranes, of the cytoskeleton, and of chromatin compaction. To prevent damage by ROS, metabolic activity is reduced and protective

molecules (antioxidants, ROS scavengers) are produced. Additionally, deposition of insoluble storage compounds and non-reducing sugars, late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs) can act as fillers and are thought to prevent protein aggregation by acting as molecular shields (for protective mechanisms see e.g. Berjak and Pammenter, 2008; Dinakar and Bartels, 2013; Farrant et al., 2007; Farrant and Moore, 2011; Gechev et al., 2012; Hoekstra et al., 2001; Terrasson et al., 2013; van Zanten et al., 2011).

The role of DT in the evolution of plants

Terrestrial organisms are constantly confronted with the desiccation stress imposed by air dryness. Consequently, during the evolution of plant life on land, adaptations that allow surviving and/or avoiding desiccation were required (Oliver et al., 2005). The ability to tolerate near complete desiccation was an important evolutionary step that played a key role in dry land colonization. Likely, DT was primitively present in chlorophytic algae that were precursors of the basal land plants (bryophytes, i.e. liverworts, mosses and hornworts) (Farrant and Moore, 2011). Bryophytes evolved mechanisms to limit water loss (cuticle and/or stomata) but the majority of bryophyte species are desiccation tolerant, which is an essential feature for life in habitats where water is not always available (Proctor et al., 2007; Proctor and Pence, 2002). Interestingly, genes responsible for synthesis and signalling of the dehydration stress hormone abscisic acid (ABA) are found in basal land plants and were likely important in the acquisition of DT and drought tolerance during plant evolution (Hauser et al., 2011; Umezawa et al., 2010). For example, a pre-treatment of the basal land plant *Marchantia polymorpha* with ABA induced protectants and resulted in morphological alterations which enhanced survival after desiccation (Akter et al., 2014). Additionally, ABA was found to regulate stomatal aperture in the model moss *Physcomitrella patens* and the lycophyte *Selaginella uncinata* (Chater et al., 2011; Ruszala et al., 2011). Further, in *P. patens*, knockouts of the *ABA INSENSITIVE 3* (*ABI3*) gene or in both class A PP2Cs (orthologs to the Arabidopsis PP2Cs that regulate ABA

signalling) were shown to affect the acquisition of DT (Khandelwal et al., 2010; Komatsu et al., 2013). In contrast to the bryophytes, higher (vascular) plants rarely have desiccation tolerant vegetative tissues. Currently, only some 330 resurrection plant species are known (<0.15% of the total number vascular plant species), which have been reported as desiccation tolerant in their vegetative parts (Proctor and Pence, 2002).

In gymnosperms (e.g. conifers), DT is completely absent from vegetative tissues, which could be explained by the ecophysiological constraint that excludes trees from being desiccation tolerant (Lüttge et al., 2011; Oliver et al., 2000). Although DT is rare in vegetative organs of angiosperms, it is present in most seeds (~95%) and pollen (~87%) of the investigated spermatophyte species (Gaff and Oliver, 2013). Basal lineages of angiosperms do not contain any desiccation tolerant plants (with DT in vegetative tissues), but they are found in later lineages, suggesting that DT in vegetative tissues was lost early during plant evolution and regained later. In fact, DT re-evolved multiple times (at least ten) in the history of angiosperms, mostly within herbaceous lineages (Oliver et al., 2000, 2005; Porembski, 2011). It is hypothesized that in these plants the activation of already present DT mechanisms (from seeds and/or pollen) were the source of genetic reprogramming for DT acquisition rather than effective adaptation of abiotic stress responses (Farrant and Moore, 2011; Gaff and Oliver, 2013; Illing et al., 2005; Oliver et al., 2000).

In spermatophytes, DT is mainly confined to seeds and pollen

It has been suggested that the rarity of DT in vegetative tissues of seed plants is related to the trade-off between DT and growth rate. Alpert (2006) discussed this trade-off along three lines of evidence: 1) from a genetic and evolutionary point of view, 2) from ecological studies that are consistent with the idea that desiccation tolerant species are poor competitors and 3) from the hypothesis that DT mechanisms constrain growth (for details see Alpert (2006) and references therein). This trade-off is visible, for example, in the moss *P. patens*. When Komatsu et al. (2013) disabled two PP2Cs that are present in the *P. patens* genome, the moss became desiccation tolerant, indicating that the PP2Cs acted as

negative regulators of an intrinsically present DT pathway. Interestingly, this double mutant was compromised in growth. Based on their data, the authors hypothesized that the PP2Cs were recruited to inhibit the DT response, allowing cells to relocate the energy once spent for DT and use it for growth and reproduction. ABA acts as environmental response signal that relieves this inhibition, releasing DT on demand upon dehydration stress.

It is likely that, as plants evolved to fill diverse niches available on dry land, the selection pressure for faster growth, plant height and dry-mass productivity (likely in combination with other water-related adaptations such as the formation of cuticle and stomata, and modifications of the root and vascular system) favoured the loss of DT in vegetative tissues (Alpert, 2005, 2000; Illing et al., 2005; Oliver et al., 2000, 2005). Although DT was lost from vegetative tissues, it was retained in reproductive propagules (seeds and pollen) (Alpert, 2006; Gaff and Oliver, 2013; Oliver et al., 2000). The confinement of DT to seeds meant an important advantage, as it enabled plants to evade a stressful season or drought period through a short life cycle or seasonal growth and survive the unfavourable period by wrapping the next generation in a stress tolerant seed.

Two traits are important for such a strategy, i.e. seed dormancy and DT. Dormancy is an important mechanism for controlling the timing of germination (to prevent plant growth during an unfavourable season/period) and increasing time for dispersal (Baskin and Baskin, 2014; Bewley et al., 2013; Finch-Savage and Leubner-Metzger, 2006). In addition, by dispersing seeds with different levels of dormancy, a plant is able to spread germination of its offspring in time, reducing the risk of losing an entire generation by a catastrophic event (Hilhorst, 2007). At the same time, desiccation tolerant seeds can remain in the soil seed bank and survive a wide range of environmental conditions. Together with dormancy, DT represents an important trait for seed survival, allowing seeds to withstand severe dehydration, as air dry tissues are very stable and able to tolerate a wide range of stressful conditions that would be detrimental to adult plants, such as extreme temperatures (Fenner and Thompson, 2005; Gaff and Oliver, 2013). There is evidence that ancestral seeds largely possessed morphophysiological dormancy

(Willis et al., 2014). Whether or not the ancestral state of seeds had DT is unclear and both possibilities have been put forward (discussed by Tweddle et al., 2003). Both traits are related to seed survival and are acquired during seed maturation, and are controlled by the plant hormone ABA. Further, the combination of dormancy and desiccation sensitivity in seeds is counterintuitive (although exceptions exist), since such seeds are generally badly storable and may die before they are able to germinate. Therefore, it seems conceivable that DT and dormancy may have evolved simultaneously. Whatever the case may be, the seed habit is an evolutionary success, to which both DT and dormancy are important contributors, as shown by the tremendous increase and diversification within seeded plants during the Cretaceous era (144-65 MY ago) and their dominance in the world's vegetation today (Linkies et al., 2010; Steeves, 1983).

DT in seeds: acquisition, loss and re-establishment

The vast majority of angiosperm species produce seeds that tolerate desiccation and long-term dry storage and are termed 'orthodox' (Roberts, 1973). Our most cultivated crops, such as rice, wheat, corn, barley, soybean and beans produce desiccation tolerant seeds. However, a significant number of wild species, particularly from wet climate areas, produce desiccation sensitive seeds. Desiccation sensitive or 'recalcitrant' seeds do not tolerate drying and are hardly storable (Roberts, 1973). Consequently, the use and conservation of recalcitrant-seeded species, which include some economically important crops, remain a challenge (Berjak and Pammenter, 2013).

Acquisition of desiccation tolerance during seed development

Seed development consists of two main phases, i.e. embryogenesis and maturation (Bewley et al., 2013). Embryogenesis comprises tissue specification and patterning, which is obtained via a well-organized series of cell divisions and cell differentiation. After its completion, seed development switches to the maturation phase that can be divided in

early and late maturation. During early maturation, the seed acquires DT and accumulates storage compounds, including proteins, oils and carbohydrates (Vertucci and Farrant, 1995). Storage of proteins in protein storage vacuoles, and lipids in oil bodies fill up the cells and offer resistance against cellular collapse upon drying (Leprince et al., 1998).

During late maturation, seeds dry out while considerable changes occur at both transcriptome and metabolome levels (Angelovici et al., 2010; Fait et al., 2006). Changes at this stage coincide with a gradual increase in seed longevity (Chatelain et al., 2012; Verdier et al., 2013). In this period, LEA proteins and non-reducing sugars, such as sucrose and raffinose family oligosaccharides, have been shown to accumulate to relatively high levels in Arabidopsis seeds (Angelovici et al., 2010; Baud et al., 2002; Hoekstra et al., 2001). LEA proteins may protect cellular structures, membranes and other proteins by acting as a hydration buffer, sequestering ions and renaturing unfolded proteins (reviewed by Tunnacliffe and Wise, 2007). Non-reducing sugars fill the free volume between large molecules, created during dehydration, allowing less molecular mobility in the matrix (Buitink and Leprince, 2004, 2008). Other structural adaptations that occur during this stage are chromatin compaction and nuclear size reduction (the latter is also observed in dried leaves of the resurrection plant *Craterostigma plantagineum*), which are reversed during germination (van Zanten et al., 2011).

Furthermore, metabolic activity is reduced towards the end of seed maturation, which minimizes the production of ROS (Pammenter and Berjak, 1999). The excessive production of ROS and a limited action of antioxidant defences can induce oxidative stress. To alleviate it, many antioxidants, such as ascorbate, glutathione, polyols, tocopherols, quinones, flavonoids and phenolics are believed to operate (Kranner and Birtić, 2005). However, to effectively limit the extent of ROS production, photosynthesis has to be down-regulated (Farrant, 2000). In seeds, the photosynthetic apparatus is usually dismantled during maturation (Bewley et al., 2013).

The expression of such protective mechanisms can be observed during both early and late seed maturation. A correct execution of the seed maturation programme is dependent on a transcriptional network referred to as the LAFL developmental network (reviewed by Jia et al.,

2014). This transcriptional network consists of master regulators that interact in a complex manner and include *LEAFY COTYLEDON* (*LEC*) *1*, *LEC2*, *FUSCA* (*FUS*) *3* and *ABI3*. *LEC1* is a HAP3 family CCAAT-box binding factor, whereas *LEC2*, *FUS3* and *ABI3* are B3 domain containing transcription factors (Giraudat et al., 1992; Lotan et al., 1998; Luerßen et al., 1998; Stone et al., 2001). Mutations in any of these genes result in severe seed maturation phenotypes. The severe *abi3-5* mutant is defective in chromatin compaction and nuclear size reduction (van Zanten et al., 2011), while a lower expression of LEA proteins has been reported for *Mtabi3* mutants (Delahaie et al., 2013). Chlorophyll breakdown is impaired in the seed maturation mutants, particularly the severe alleles of *abi3* in which chlorophyll degradation does not take place and mature seed possesses green cotyledons (Delmas et al., 2013; Nambara et al., 1992, 1995; Ooms et al., 1993). A lack of chlorophyll degradation was also found in two *Mtabi3* mutants in Medicago (Delahaie et al., 2013). These seed maturation mutants failed to acquire DT and were barely storable. For example, *abi3-5* and *lec1-3* were shown to have a severely reduced number of germinating seeds at harvest (due to a lack of DT) and displayed a strongly reduced longevity (Sugliani et al., 2009). Besides the important roles of *ABI3* and *LEC1*, the strong seed maturation phenotypes of these mutant alleles were shown to be affected by the genetic background. Introgressions of the accessions of Seis am Schlern (Sei-0) and Shahdara (Sha-0) partially suppressed the *abi3-5* and *lec1-3* phenotypes and thus allowed the identification of genetic loci that could improve seed longevity (Sugliani et al., 2009). It is not possible to establish such improved longevity in seeds that do not tolerate desiccation, indicating that these loci also control the acquisition of DT (at least to a certain extent) in these severe maturation mutants. Thus, the identified loci are important for the acquisition of DT as well as seed longevity.

A window of desiccation tolerance in germinated seeds

DT is usually fully established just before the drying phase, towards the end of seed maturation and is generally lost during germination (Bewley et al., 2013). Drying back of seeds at different intervals along

the germination-time curve has shown that seeds were killed already before or quickly after visible germination (Buitink et al., 2003; Daws et al., 2007a; Lin et al., 1998; Maia et al., 2011; Vertucci and Farrant, 1995). It should be noted that these analyses were based on fast drying treatments in which seeds lose most of their water within ~2 h. However, germinated seeds have actually a longer window in which they tolerate desiccation. The existence of this window can be observed when a mild osmotic stress (by a polyethylene glycol (PEG) treatment) is applied before fast drying. This has been demonstrated for a number of species, including *Cucumis sativus*, *Impatiens walleriana*, Medicago, *Tabebuia impetiginosa* (Brazilian tree species), and, recently, Arabidopsis (Bruggink and van der Toorn, 1995; Buitink et al., 2003; Maia et al., 2011, 2014; Vieira et al., 2010). For this reason, the mild osmotic stress is said to re-induce DT in germinated seeds. Nevertheless, at a certain developmental stage after germination, orthodox seeds completely lose the ability to tolerate extreme drying (even after application of a mild osmotic stress) and become desiccation sensitive (Fig 2.2). Thus, this ability is strictly dependent on the developmental stage of the germinated seeds (Buitink et al., 2003; Leprince et al., 2000; Maia et al., 2011; Vieira et al., 2010). For example, Medicago seeds with radicle length up to 1 mm survive fast drying. When the radicles are up to 2.7 mm in length, their ability to become fully desiccation tolerant is dependent on a mild osmotic stress treatment using PEG (Buitink et al., 2003).

To determine the developmental window in which germinated Arabidopsis seeds could be triggered to re-induce DT, four clearly distinct developmental stages were defined (Maia et al., 2011, 2014): (I) at testa rupture, (II) at radicle protrusion, (III) with a primary root of 0.3 – 0.5 mm in length, and (IV) at the appearance of the first root hairs (Fig 2.2). In the first three stages, seeds were able to withstand desiccation after a PEG treatment and seedling survival rates were close to 100%. At stage IV, this number dropped to ~20-40% indicating that this ability was largely lost at this stage. Another noticeable feature was that different seed parts displayed variable levels of re-induction of DT. In Arabidopsis, the cotyledons were the most tolerant tissue followed by hypocotyls and roots. Also in Medicago, the cotyledons are more tolerant to desiccation as compared to the radicles (Buitink et al., 2003).

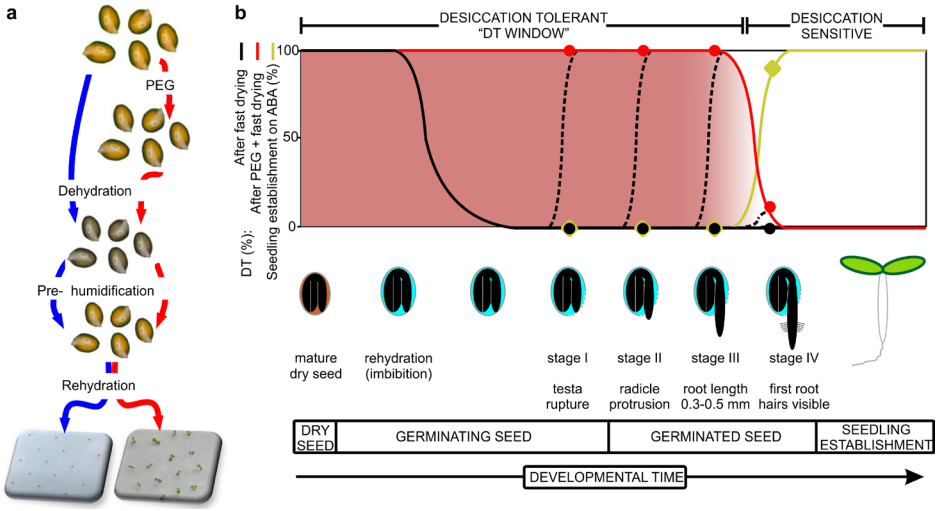


Fig 2.2 *Loss of DT in germinating seeds.* a *Schematic representation of the re-induction of DT by PEG treatment. When germinated seeds at the stage of radicle protrusion (stage II) are fast dried (*blue arrows*), they do not survive, as shown by the lack of seedling growth after pre-humidification (to reduce imbibitional damage) and rehydration. In case the drying treatment is preceded by a 3 d PEG treatment, seeds survive, as shown by further growth and seedling establishment.* b *Schematic representation of the DT window. Arabidopsis seeds at testa rupture stage (stage I) do not survive fast desiccation treatment and resistance against this treatment is already lost earlier during imbibition (*black solid line*). However, a PEG treatment is able to re-induce tolerance against this desiccation treatment in stages I to III but this is largely lost at stage IV (as indicated by the* dashed lines*). The* red line *shows DT after PEG treatment and indicates the DT window after germination. This window strongly correlates with ABA sensitivity (*green line*) which is also restricted to a limited time window after germination (Lopez-Molina et al. 2001; Maia et al. 2014).*

These experiments revealed a developmental window in which germinated seeds have the capacity to tolerate desiccation, helping to differentiate between stages in which desiccation is tolerated (the DT window) or is completely lost (as is shown for Arabidopsis in Fig 2.2). This DT window represents an extreme stress tolerance mechanism that could be ecologically relevant for seedling establishment. The acquisition of DT is an active process, which takes some time to become effective. Interestingly, the observation that DT can be re-induced in germinated seeds by PEG treatment resembles observations made in mosses. Of 62 moss species studied, 22% showed a DT phenotype, but this was increased to 71% in case these same moss species were hardened

before desiccation (Gaff and Oliver, 2013).

The loss of DT has been correlated with the start of cell division (Boubriak et al., 2000; Faria et al., 2005; Osborne and Boubriak, 1994). The switch from desiccation tolerant to desiccation sensitive coincides with radicle cells entering the G2 phase of the cell cycle, which contains the double amount of DNA (Faria et al., 2005; Saracco et al., 1995). This could potentially affect the induction of DT. In tomato seeds, DNA synthesis precedes germination, which does not support such correlation. Thus, whether DNA duplication or cell cycle activation are the key triggers for the loss of DT remains questionable. In tomato for example, seed priming results in DNA synthesis in non-germinated seeds (Bino et al., 1992) and reduction of seed longevity, but does not lead to loss of DT. Furthermore, Arabidopsis mutants affected in DNA repair (DNA ligase enzymes, *atlig4* and *atlig6*) displayed reduced seed longevity (Waterworth et al., 2010). Also microtubular dynamics and integrity are affected by dehydration (Sargent and Osborne, 1981) and could be related to the loss of DT. Interestingly, the ability of yeast to tolerate desiccation is related to growth rate (and thus cell division). Within the cells that are growing exponentially, only one in a million survives desiccation whereas 1 in 5 survives desiccation in the stationary phase (Calahan et al., 2011; Welch et al., 2013). Survival can be enhanced in exponentially growing cells by exposure to heat stress or nutrient limitation before desiccation. These treatments alter growth rate which is probably correlated with the reduction of 60S ribosomal subunit biogenesis (Welch et al., 2013). Yeast mutants that are affected in 60S biogenesis have an increased DT. Whether DT in plants is also regulated at the level of ribosomal subunit biogenesis remains to be shown.

The ability to withstand drying coincides with structural changes in chromatin compaction and nuclear size reduction (van Zanten et al., 2011) and these structural adaptations are not reverted upon rehydration alone. The nuclear size in rehydrated dormant seeds remains small. In non-dormant seeds, the reversion to a larger nuclear size seems related to germination and the largest increase is observed between 2 and 3 days after sowing (van Zanten et al., 2011). Although several processes are correlated with the loss of DT in germinated seeds, its genetic regulation and the molecular mechanisms involved are still poorly understood.

Regulation of DT in seeds

Transcriptional regulation of desiccation tolerance

The re-induction of DT in Medicago radicles was studied by using transcriptome profiling. Three-millimetre-long radicles are sensitive to fast drying but this can be reverted by a PEG treatment. A time course of gene expression profiling revealed ~1,300 differentially expressed genes during the re-induction of DT in PEG-treated radicles of Medicago (Buitink et al., 2006). Most of these genes (~720) were down regulated and related to cell cycle, biogenesis, and primary metabolism. Sucrose accumulates in desiccation tolerant radicles and a combination of transcriptome and metabolite measurements indicates that sucrose is produced by mobilizing lipids and starch (Buitink et al., 2006). Also, LEAs are rapidly induced transcriptionally and their accumulation was confirmed by analysis of the heat stable proteome (Boudet et al., 2006). Interestingly, a significant overlap was found between genes that are differentially expressed during seed maturation (between 14 and 20 DAP) and during the re-induction of DT in radicles (Buitink et al., 2006; Terrasson et al., 2013). Based on these transcriptome data, it appeared that during the re-induction of DT, germinated seeds (partially) revert to an earlier developmental stage (Buitink et al., 2006). In Arabidopsis, the top 50 DT up-regulated genes during re-induction of DT were down-regulated during germination, while the top 50 DT down-regulated showed generally an increased expression during germination, supporting this 'reversion' theory (Maia et al., 2011).

Also other transcriptome data show that the largest set of differentially expressed genes is down-regulated during re-induction of DT (2,829 down vs 740 up, Terrasson et al. (2013) and 414 down vs 263 up, Maia et al. (2011), for Medicago and Arabidopsis, respectively). This might indicate the importance of shutting down certain processes as part of the ability to induce DT. Although the specific overrepresented GO terms differed between both species, the genes in both down-regulated sets related to cellular metabolic processes, biogenesis, and growth. Additionally, in Arabidopsis also photosynthesis-related genes were down-regulated. The up-regulated gene classes revealed a larger

overlap between the GO terms found in Medicago and Arabidopsis, and include response to stress, response to abiotic stimulus, response to water deprivation, response to abscisic acid stimulus, lipid localisation, seed development, and embryonic development ending in seed dormancy (Maia et al., 2011; Terrasson et al., 2013).

Another powerful approach to obtain insights into the regulation and downstream processes involved in DT was taken by the construction of a coexpression network (Verdier et al., 2013). In this network, several modules that linked gene expression to different processes of seed development, including embryogenesis, seed filling, DT, and final maturation drying were identified. The DT module contained genes related to stress responses, LEAs and ABA-induced genes. Another gene regulatory network containing 22 seed specific transcription factors and seed specific probe sets indicated genes that correlate strongly with DT, LEAs, and longevity (Verdier et al., 2013). Four transcription factors, *MtABI3* (Giraudat et al., 1992; Koornneef et al., 1984; McCarty et al., 1989), *MtABI4* (Finkelstein, 1994; Finkelstein et al., 1998), *MtABI5* (Finkelstein, 1994; Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000) and an *MtAP2/EREBP* gene, were found to be highly connected with DT genes, and are therefore good candidates as DT regulators. *MtABI3* is one of the most connected transcription factors. Interestingly, a large proportion of the genes connected to *MtABI3* in the network were identified as direct targets of ABI3 in Arabidopsis (Mönke et al., 2012; Verdier et al., 2013).

Role for ABA signalling in desiccation tolerance

The phytohormone ABA is a central regulator of plant development and responses to environmental stresses. Currently, over 100 loci have been identified as being involved in ABA perception and downstream signalling (Cutler et al., 2010). ABA controls seed developmental processes, including accumulation of food reserves, as well as acquisition of dormancy and DT (Kermode and Finch-Savage, 2002). In Arabidopsis, DT is acquired slightly later during seed maturation in the ABA-deficient mutant *aba1-1* as compared to the wild type (Koornneef et al., 1989). It is likely that *aba* mutants in Arabidopsis are not complete null mutants,

as discussed by Barrero et al. (2005) based on their analysis of several mutant alleles of *ABA1* as well as double mutant analysis with other ABA deficient mutants (*aba2* and *aba3*). Therefore, they suggested that an alternative route might produce minor amounts of ABA (Barrero et al., 2005). Possibly, such low levels of ABA are enough to evoke DT. This is supported by the finding that seed specific expression of an ABA antibody in tobacco (*Nicotiana tabacum*) resulted in much stronger seed phenotypes including desiccation sensitive seeds (Phillips et al., 1997). In Arabidopsis, the triple mutant of SnRK2 genes (*snrk2.2/3/6*) is severely distorted in ABA signalling. Although these mutant seeds germinate just after harvest, following drying, their germinability is already reduced after one week and lost after two weeks of dry storage, indicating a severe seed longevity lesion (Nakashima et al., 2009).

As mentioned before, in germinated seeds, DT can be re-induced by a mild osmotic treatment using PEG. This response can be mimicked by applying ABA instead, as has been shown in *C. sativus*, Medicago and Arabidopsis (Buitink et al., 2003; Lin et al., 1998; Maia et al., 2014). In germinated seeds, the induction of DT depends on ABA. Fluridone (an ABA biosynthesis inhibitor) treatment of Medicago radicles and the use of the Arabidopsis *aba2-1* mutant (disrupted in ABA biosynthesis) compromised the re-induction of DT (Buitink et al., 2003; Maia et al., 2014). In germinated Arabidopsis seeds the osmotic treatment did not appear to change the ABA content of the seeds, but, likely, influenced sensitivity to ABA. Interestingly, two ABA receptors (i.e. *PYL7* and *PYL9*) were highly induced upon PEG treatment (Maia et al., 2014).

In agreement with a role of ABA, several mutants in ABA signalling were shown to be compromised in their ability to re-induce DT in germinated seeds. For example, two *abi5* mutants, *Mtabi5-1* and *Mtabi5-2*, lacked the ability to re-induce DT in Medicago radicles by osmotic stress (Terrasson et al., 2013). Maia et al. (2014) reported phenotypes in the re-induction of DT in germinated seeds for several mutants in ABA signalling, such as *abi3-8*, *abi3-9*, *abi4-3* and *abi5-7*. In spite of being compromised for ABA sensitivity or synthesis, all of the mutants tested produced desiccation tolerant seeds at the end of seed maturation. However, all five mutants showed a reduced capacity to re-induce DT in germinated seeds. These observations imply that the acquisition of

DT during seed development is different from the re-induction of DT in geminated seeds. There are two hypotheses to explain these differences. First, except for ABI3 (that is clearly involved in both), largely distinct pathways are involved in the induction of DT during seed development and following germination. Second, the pathways that induce DT involve ABA, *ABI4*, and *ABI5*, but their function remains unnoticed when testing the mutant alleles, because of additional redundant factors present during seed development but absent after germination. In the gene regulatory network presented by Verdier et al. (2013), *ABI3*, *ABI4*, and *ABI5* are strongly linked to DT genes supporting the latter hypothesis.

Lopez-Molina et al. (2001) have identified a small developmental window of ABA sensitivity after germination, in which seedling growth could be arrested. Such arrested seedlings were resistant to a drying treatment. It has been suggested that during this phase, the young plantlets monitor the environmental osmotic status. In case of dehydration stress, ABA, via ABI3 and ABI5, induces a developmental arrest of germinated embryos, thereby protecting young seedlings from the loss of water (Llorente et al., 2002; Lopez-Molina et al., 2001). Interestingly, the DT window overlaps with this ABA sensitivity window: DT could be re-induced in Arabidopsis seeds at developmental stages I to III, when ABA sensitivity was high, whereas low ABA sensitivity levels at stage IV correlated with a reduced ability to re-induce DT (Fig 2.2) (Maia et al., 2014). This supports the hypothesis above and confirms the importance of ABA in DT induction (Buitink et al., 2003; Maia et al., 2014; Terrasson et al., 2013; Verdier et al., 2013). Thus, seedling establishment might not only be regulated by control of germination, but by an additional post-germination checkpoint as well (Llorente et al., 2002; Lopez-Molina et al., 2001).

A role for sugar signalling in desiccation tolerance?

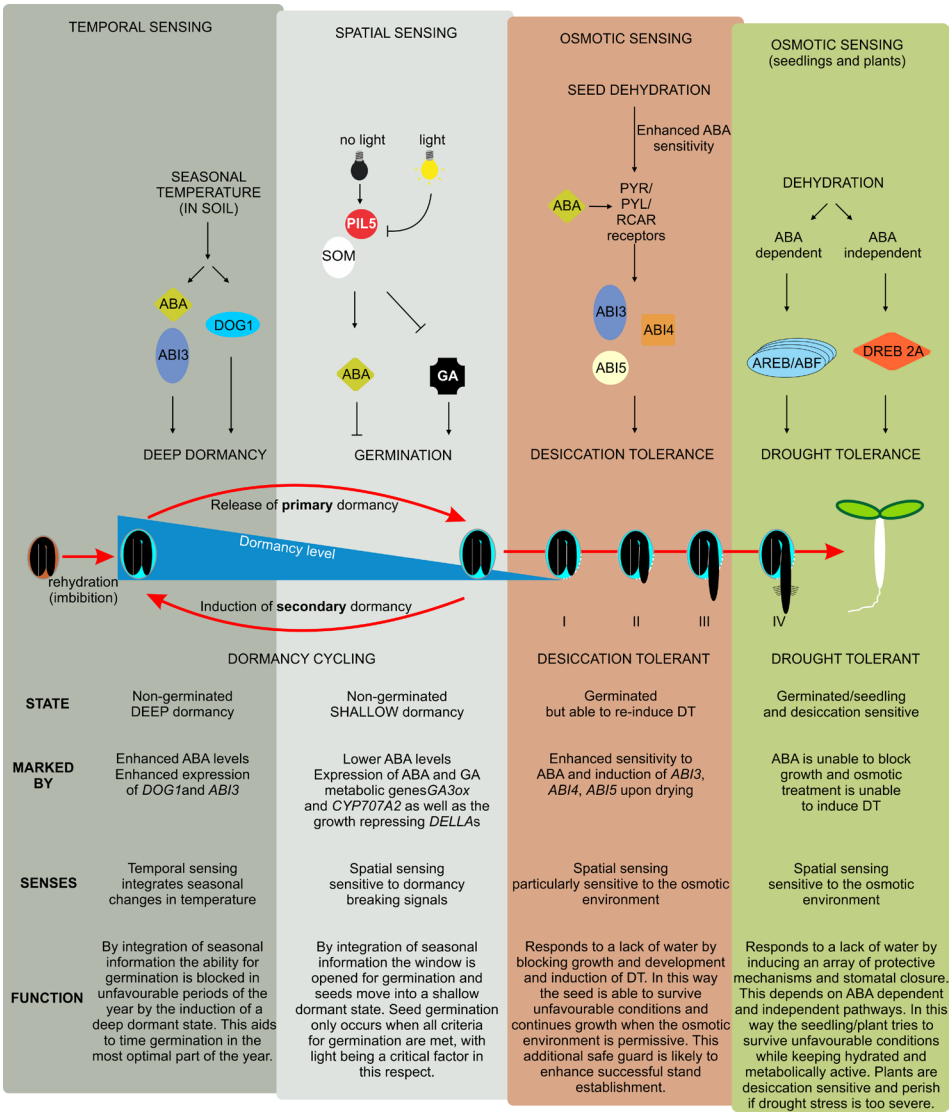
In addition to the role of sugars as protectants, high exogenous levels of sugars are able to arrest seedling establishment in an ABA-dependent manner (Dekkers and Smeekens, 2007). Both ABA and sugar arrested seedlings have an increased resistance to a drying treatment (Dekkers et al., 2008; Lopez-Molina et al., 2001). Germinated seeds are

sensitive to sugar-induced arrest during an approximately 2-3 day time-window following germination (Gibson et al., 2001). In this developmental window, embryonic marker genes like *ABI3*, *ABI5* and several *LEA* genes can be induced by sugars (Dekkers et al., 2008). In the liverwort *M. polymorpha*, ABA-induced survival after a desiccation treatment was strongly promoted in the presence of sugars (Akter et al., 2014). The addition of glucosamine (an inhibitor of hexokinase) early during the PEG treatment negatively affected the ability to re-induce DT in Medicago and *C. sativus* radicles (Leprince et al., 2004). The HEXOKINASE 1 protein in Arabidopsis was shown to possess a dual function, acting in signaling and as an enzyme (Moore et al., 2003). Currently it is unclear whether the effect of glucosamine in the re-induction of DT is caused by the repression of enzymatic activity or sugar signaling. Thus, possibly, sugars play a dual role by acting both as structural protectants (as mentioned before) and signaling intermediates. With having Arabidopsis as an experimental model for studies on the re-induction of DT, all the genetic resources available for this species can be used to address these questions.

Combinatorial roles of dormancy and the DT window in optimizing seedling establishment?

Obviously, the establishment of seedlings is strongly affected by the control of germination via dormancy mechanisms that control when, and the conditions in which, germination occurs. The DT/ABA window might represent an additional layer of control. It capacitates germinated seeds to arrest development and survive a period of water limitation by complete desiccation and thereby optimize seedling establishment. Thus, seedling establishment is regulated at least at three different levels: at the germination level via *temporal* and *spatial sensing* and at the level of the ABA/DT window (see Fig 2.3). These regulation mechanisms are discussed in more detail below.

To achieve germination in the proper period of the year and under



favourable conditions, seeds sense their environment at two levels, which are referred to as temporal and spatial sensing. Within a year, seeds can alternate between the deep and shallow dormancy which, basically, opens and closes the window in which seed germination can occur (Bouwmeester and Karssen, 1993; Derkx and Karssen, 1994; Footitt et al., 2011; Hilhorst and Karssen, 1988). Seeds achieve this by integrating slow seasonal changes, via temporal sensing of, most likely,

◀ Fig 2.3 *Seedling establishment is regulated on different levels by seed dormancy and DT. Low temperatures over winter (by temporal sensing of seasonal changes) induce strong dormancy and close the germination window in Cvi seeds (Footitt et al., 2011). This is linked with enhanced levels of ABA, and higher expression of* ABI3 *and* DOG1*. Release of primary dormancy occurs gradually and results in a more shallow state of dormancy. At different stages, seeds become sensitive to dormancy breaking (Finch-Savage et al., 2007; Finch-Savage and Leubner-Metzger, 2006). Sensing of environmental conditions during this shallow dormant state is referred to as spatial sensing in which light is a critical factor for germination (Footitt et al., 2011). In the absence of light, germination is inhibited by the activity of* PIL5 *(Oh et al., 2004).* PIL5 *stimulates the expression of a CCCH-type zinc finger gene called* SOMNUS *(*SOM*) (Kim et al., 2008). Upon a light signal* PIL5 *repression is relieved which allows seed germination to occur. After germination, in stage I – III, seeds are able to survive complete dehydration which represents an important mechanism to an otherwise deadly stress and likely helps to optimize successful seedling establishment. The induction of DT at this stage relies on ABA and three transcription factors that play an important role in ABA signalling (ABI3, ABI4, and ABI5). From stage IV onwards, germinated seeds largely lost the ability to tolerate desiccation. Likely, the response switches to drought tolerance instead, which is regulated by ABA dependent as well as independent pathways (for a recent review see Yoshida et al., 2014).*

temperature (Bouwmeester and Karssen, 1993), which sets the depth of dormancy. During shallow dormancy, when seeds are able to germinate if the environmental conditions permit it, seeds can respond fast (by spatial sensing) to favourable germination conditions (Footitt et al., 2011). The presence of light and its spectrum are critical signals in this respect and likely act as a gap-detection mechanism or as sensor to ensure that the seed, once germinated, is close enough to the soil surface for successful seedling establishment (Bewley et al., 2013; Footitt et al., 2011; Silvertown, 1980). In the absence of light, the activity of *SOMNUS* (*SOM*) and of the bHLH transcription factor PIF3-LIKE (PIL) 5 inhibit germination (Kim et al., 2008; Oh et al., 2004). These proteins affect genes involved in ABA and gibberellin metabolism and signalling and both *som* and *pil5* mutants germinate in the absence of light (Kim et al., 2008; Oh et al., 2006). If during this stage the proper environmental conditions are not met to complete germination, the window for germination closes by the induction of a deeper state of (secondary) dormancy through temporal sensing (Fig 2.3). However, light relieves PIL5 repression which interrupts the germination repressing circuitry and thereby promotes germination (see Fig 2.3 for a simplified scheme).

Dormancy, through temporal sensing, ensures germination in the right time of the year and, through spatial sensing, ensures that germination only occurs under the right circumstances. Although these are very important mechanisms, the decision (upon spatial sensing) of a seed to germinate is based on a snapshot of the environment (light, water, and temperature, for example) that is favourable. However, the environmental conditions (weather) can be unpredictable and seeds can encounter serious stresses once germinated, including lack of water. The fact that germinated seeds have a window in which they cope with dehydration by tolerating the stress is likely beneficial. Thus, in response to the lack of water, germinating or germinated seeds are able to stop growth and remain in a quiescent desiccated state only to resume development when water is in ample supply again. This response could represent an ecological important stress tolerance mechanism that optimizes successful seedling establishment in conjunction with dormancy, as explained above (Fig 2.3). When this ability is lost and seedlings become irreversibly desiccation sensitive, Arabidopsis roots have formed root hairs already. Root hairs grow from specialized epidermal cells (trichoblasts) and are important structures for water uptake and anchoring in the soil (Gilroy and Jones, 2000). This observation suggests that the switch from desiccation tolerant to desiccation sensitive in Arabidopsis is made around the point when the germinated seed is capacitated for anchoring and active water uptake from its environment by these specialized structures.

The loss of DT may underlie a change in the response to dehydration, which is switched from *desiccation* tolerance (in stage I-III in Arabidopsis) to *drought* tolerance (from stage IV onwards). Although several factors have been related to the loss of DT (like start of cell division), this switch is not well understood. Since the ability of Arabidopsis seeds to re-induce DT during germination is tightly linked with ABA sensitivity, this switch could be linked with a change in ABA response or sensitivity. The ABA mode of action might change during this developmental switch from a DT to a drought-tolerance inducing agent. During the first three stages (Maia et al., 2011), ABA induces a stress-tolerant state by blocking growth and inducing quiescence and essential mechanisms to re-induce DT. However, from stage IV onwards the seedling attempts to limit the

loss of water and thereby staying hydrated and metabolically active, employing the ABA signal to induce cellular protective and water saving mechanisms (drought tolerance). In Arabidopsis, ABA re-induces late embryogenesis related genes (*AtEM1, AtEM6, RAB18*) within the ABA-sensitive window (DT window). Beyond this window, ABA fails to block growth and re-induction of these late embryogenesis related genes. Instead, other stress related genes such as *COR47* and *RD29A* are activated (Llorente et al., 2002). In Medicago, radicles that are 5 mm in length are unable to regain DT after a PEG treatment. However, such PEG-treated radicles tolerate dehydration much better compared to untreated radicles, as was shown by a survival curve. It showed that 50% survival was obtained at 0.8 g H_2O/g DW for PEG-treated radicles compared to 3.6 g H_2O/g DW for the untreated ones (Boudet et al., 2006). Combined, these data suggest that during the progression of radicle elongation the response towards water limitation shifts from DT to drought tolerance. Since ABA does not block growth and does not induce quiescence as it does during the induction of DT, this could explain the 'ABA insensitive' phenotype of germinated seeds in stage IV. This developmental switch likely involves chromatin remodelling factors like PICKLE and histone deacetylases, that repress either embryonic or seedling traits (Perruc et al., 2007; Tanaka et al., 2008; van Zanten et al., 2011). Moreover, given the essential role of ABA in DT induction, a reduction in ABA sensitivity after visible germination could be a critical factor to reduce the ability to re-induce DT. Rubio et al. (2009) described a triple mutant of three PP2C genes (which are negative regulators of ABA signalling) that showed a strong growth reduction, extreme sensitivity to ABA, delayed germination, and a partially constitutive ABA responsive transcriptome. Analysis of such a genotype could uncover whether desensitizing of ABA signalling is involved in the regulation of this developmental switch.

Conclusion and future perspectives

DT has been a critical trait during the evolution of plants on land. In most angiosperm species, vegetative DT has been lost, although the

majority of species maintained this trait in their seeds. This already indicates the important role of DT in seed function. Medicago has been used as a model for over a decade in the study of DT in seeds. The system in which DT is lost in germinated seeds and re-induced by an osmotic treatment has proven to be a powerful approach. More recently, germinated Arabidopsis seeds have also emerged as a strong model to investigate the re-induction of DT. Recent results confirmed the role of ABA and ABI3 in the acquisition of DT and supported a role for ABI4 and ABI5 in this process. The results of both model systems are complementary and suggest at least a certain level of conservation in the re-induction of DT in germinated seeds.

An ABA sensitivity window was proposed as a post-germination check-point of the osmotic environment during germination towards seedling establishment and was suggested to be related to DT (Lopez-Molina et al., 2001, 2002). The observation that the DT window overlaps with this ABA sensitivity window strongly supports this hypothesis. Thus, seedling establishment is controlled, on the one hand by dormancy (by timing germination through temporal and spatial sensing) and on the other hand by a post-germination window in which growth can be blocked in the absence of water and resumed when osmotic conditions become favourable (Fig 2.3).

Whether this DT window is indeed important for seedling establishment and reproductive success remains to be proven and several issues remain to be clarified. E.g., to be an effective stress tolerance mechanism during and after seed germination, DT should be able to be induced under natural drying conditions that occur in the soil and the dried seeds should be viable for a certain period of time. Importantly, recent research in Medicago and Arabidopsis identified several genotypes that are disturbed in re-induction of DT in germinated seeds, and perhaps these might be useful to answer the question whether this DT window plays an important role in seedling establishment under suboptimal conditions. Alternatively, such genotypes can be sown in the field to assess the importance of this DT window in seedling establishment and reproductive success under field conditions. Research in these directions may provide a further understanding of the ABA/DT developmental window present in germinated seeds.

Furthermore, the regulation underlying the developmental switch from DT (ABA sensitive) to drought tolerance (ABA "insensitive") is not well understood and needs further study. Also, there is little information whether natural variation exists for this trait. Perhaps variation exists for the sensitivity to induce DT in germinated seeds or in the length of the window in which this is possible. Such variation (if present), together with a better understanding of this ABA/DT window, and its regulation, may offer future possibilities to improve stand establishment in field grown crops.

Acknowledgements

This work was supported by the Netherlands Organization for Scientific Research (NWO), the Dutch Technology Foundation, which is the applied science division of the Netherlands Organization for Scientific Research and the Technology Program of the Ministry of Economic Affairs; by the 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior' (CAPES, Brazil); and by the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Brazil).

Chapter
Three

A gene co-expression network predicts functional genes controlling the re-establishment of desiccation-tolerance in germinated *Arabidopsis thaliana* seeds

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Published as Costa et al., (2015) Planta 242:435-449

Abstract

Main conclusion During re-establishment of desiccation tolerance (DT), early events promote initial protection and growth arrest, while late events promote stress adaptation and contribute to survival in the dry state.

Mature seeds of *Arabidopsis thaliana* are desiccation tolerant, but they lose desiccation tolerance (DT) while progressing to germination. Yet, there is a small developmental window during which DT can be rescued by treatment with abscisic acid (ABA). To gain temporal resolution and identify relevant genes in this process, data from a time series of microarrays were used to build a gene co-expression network. The network has two regions, namely Early Response (ER) and Late Response (LR). Genes in the ER region are related to biological processes, such as dormancy, acquisition of DT and drought, amplification of signals, growth arrest and induction of protection mechanisms (such as LEA proteins). Genes in the LR region lead to inhibition of photosynthesis and primary metabolism, promote adaptation to stress conditions and contribute to seed longevity. Phenotyping of 12 hubs in relation to re-establishment of DT with T-DNA insertion lines indicated a significant increase in the ability to re-establish DT compared with the wild-type in the lines *cbsx4*, *at3g53040* and *at4g25580*, suggesting the operation of redundant and compensatory mechanisms. Moreover, we show that re-establishment of DT by polyethylene glycol and ABA occurs through partially overlapping mechanisms. Our data confirm that co-expression network analysis is a valid approach to examine data from time series of transcriptome analysis, as it provides promising insights into biologically relevant relations that help to generate new information about the roles of certain genes for DT.

Keywords

ABA, ABI3, LEA proteins, Transcriptome

Introduction

During seed development, orthodox seeds acquire the remarkable ability to tolerate desiccation. This means that during development, as a part of the maturation phase, these seeds experience slow reduction of their water content to less than 5% of their dry weight with little or no loss of viability (Ooms et al., 1993). In this dry state, orthodox seeds can survive for years or centuries, which permits their storage and ensures better dispersal (Ramanjulu and Bartels, 2002). During seed imbibition and germination, increasing water availability allows metabolic processes to resume, eventually leading to the emergence of the radicle and the associated progressive loss of desiccation tolerance (DT) (Buitink et al., 2003, 2006; Maia et al., 2011). Yet, after radicle protrusion, there is a small developmental window during which DT can be rescued by treatment with an osmoticum (polyethylene glycol, PEG) and/or the plant hormone abscisic acid (ABA) (Buitink et al., 2003, 2006; Maia et al., 2011, 2014).

Incubation in PEG induces membrane changes, inhibits radicle growth, down-regulates genes related to energy metabolism and cell wall modification, up-regulates genes related to antioxidant activity, response to stress and seed storage, and induces synthesis of protective molecules, such as non-reducing sugars and certain proteins, such as late embryogenesis abundant (LEA) and heat shock proteins (HSPs) (Buitink et al., 2003, 2006; Maia et al., 2011). Furthermore, genes coding for ABA signal transduction elements and drought/stress induced transcription factors (TFs) are also up-regulated, leading to the hypothesis of a partial overlap of ABA-dependent and -independent regulatory pathways involved in both drought and DT (Buitink et al., 2003, 2006; Maia et al., 2011). Mutants compromised in ABA sensitivity or synthesis were shown to be still able to produce desiccation tolerant seeds that, however, are impaired in re-establishment of DT during germination (Maia et al., 2014). Moreover, ABA perception and signalling, more than ABA content, are influencing the ability to re-establish DT (Maia et al., 2014). In previous studies, the re-establishment of DT was brought about by the simultaneous application of cold and osmotic stress (Buitink et al.,

2003), or osmotic stress and/or ABA (Maia et al., 2011, 2014). Although treatment of germinated seeds with ABA alone is able to re-establish DT (Maia et al., 2014), the regulatory and mechanistic pathways activated via ABA remain to be elucidated.

Here, we used microarrays to characterize global gene expression during the re-establishment of DT in germinated *A. thaliana* seeds by ABA, and used the data for network analysis of gene co-expression, in order to gain more resolution on and identify relevant genes in this process. Gene-expression data derived from microarray analysis have been commonly used to provide insight into biological processes at a system-wide level (Freeman et al., 2007; Wang et al., 2006) and are well-suited to reconstruct gene regulatory networks to explore potential biological relations (Wang et al., 2006). These networks consist of nodes that represent genes connected by edges that infer co-expression based on a correlation threshold (Freeman et al., 2007). The analysis of this kind of networks is based on the assumption that genes with similar expression patterns have similar functions (Freeman et al., 2007).

In this study, the construction and analysis of a co-expression network proved to be a valid approach to examine data from microarrays of a time series of DT re-establishment by ABA. Using a combination of physiology and transcriptomic approaches, we generated and partially validated a co-expression network, which revealed two major patterns of gene expression during the re-establishment of DT by ABA: an early and a late response. Genes could be divided in those that respond earlier to the incubation in ABA and provide initial protection and signal transduction, and those that respond later and provide adaptation to the stress condition. Validation of the network by functional characterization of genes identified based on the network analysis revealed important roles of some of these genes in the re-establishment of DT in germinated seeds.

Materials and methods

Plant growth conditions and germination assays

Arabidopsis thaliana plants, accession Columbia (Col-0, N60000), were grown on Rockwool plugs (MM40/40; Grodan), in a climate cell (20˚C day, 18˚C night), under 16 h of light, and watered with Hyponex nutrient solution (1 g l^{-1}, http://www.hyponex.co.jp). Seeds were bulk harvested in three replicates of at least two plants. Seeds used in germination assays were cold stratified for 72 h at 4˚C in 9-cm Petri dishes on two layers of blue filter paper (Blue Blotter Paper, Anchor Paper Company, http://www.seedpaper.com) and 10 ml of distilled water. After stratification, seeds were transferred to germination cabinets with constant white light at 22˚C.

Re-establishment of DT using ABA

To assess the re-establishment of DT using ABA, germinated seeds at the stage of radicle protrusion (stage II, Maia et al., 2011) were incubated for a maximum of 3 days in 6-cm Petri dishes containing 1.3 ml of an ABA solution (10 µM) on two sheets of white filter paper (grade 3 hw, Biolab Products, Sartorius Stedim Biotec) in the dark at 20˚C. The incubation in ABA was done in the dark in order to reduce oxidative damage. After incubation, seeds were rinsed in distilled water, transferred to new Petri dishes with one dry sheet of white filter paper and dried for 3 days at 40% relative humidity (RH) at 20˚C, resulting in water content levels as low as 0.08 g H_2O g^{-1} dry weight. After drying, seeds were pre-humidified in air of 100% RH for 24 h at 22˚C in the dark and subsequently rehydrated in water on a Copenhagen Table under a 12/12 h dark/light regime at 20˚C. Germinated seeds were evaluated according to the survival of their primary root, presence of green and fully expanded cotyledons (cotyledon survival) and growth resumption with both green and fully expanded cotyledons and development of a root system (seedling survival).

Longevity of germinated seeds

Longevity of germinated seeds was evaluated by an accelerated ageing test after incubation in ABA for 24 h and 72 h, drying for 3 days at 40% RH, storage for 24 h at 80% RH and 40˚C in the dark and rehydration. The parameters evaluated were survival of the primary root, cotyledons survival and seedling survival.

RNA extraction and microarray hybridization

Seeds at the stage of radicle protrusion (control) and these seeds after four periods (2 h, 12 h, 24 h and 72 h) of incubation in ABA were used for RNA extraction. Total RNA was extracted from three replicates of approximately 1,000 germinated seeds for each time point following a modified hot borate protocol (Maia et al., 2011; Wan and Wilkins, 1994). The seeds were ground and mixed with 800 µl of extraction buffer (0.2 N Na borate decahydrate (Borax), 30 mM EGTA, 1% SDS, 1% Na deoxycolate) containing 1.76 mg DTT and 52.8 mg PVP40, and heated to 80˚C. In the next step, 4 mg proteinase K was added to this solution before incubation for 15 min at 42˚C. After the addition of 64 µl of 2 M KCL, the samples were incubated on ice for 30 min and subsequently centrifuged for 20 min at 12,000 *g*. The supernatant was transferred to a new tube, 260 µl of ice-cold 8 M LiCl was added, and the tubes were incubated overnight on ice. After centrifugation at 4˚C for 20 min at 12,000 *g*, the pellets were washed with 750 ml of ice-cold 2 M LiCl and re-suspended in 100 µl milliQ water. The samples were phenol-chloroform extracted, DNAse treated (RQ1 DNase, Promega) and further purified with RNeasy spin columns (Qiagen) according to the manufacturer's instructions. RNA quality and concentration were assessed by agarose gel electrophoresis and a NanoDrop ND-1000 spectophotometer (Nanodrop® Technologies, Wilmington, DE, USA). RNA was processed for the use on Affymetrix ARAGene 1.1ST Arrays as described by the manufacturer. In brief, reverse transcription was employed to generate double-stranded cDNA that was in vitro transcribed to biotinylated cRNA. The biotinylated cRNA was used for hybridization. The Affymetrix HWS Kit for GeneTitan (part nr. 901622) was used for hybridization, washing

and staining of the array plates. The array plates were scanned using the Affymetrix Command Console v3.2 software.

All data are MIAME compliant as detailed on the MGED Society website http://www.mged.org/Workgroups/MIAME/miame.html. The microarray data has been deposited on the NCBI's Gene Expression Omnibus (Edgar et al., 2002) and is accessible through the GEO Series accession number GSE62876.

Microarray analysis

Signal intensities were extracted and analysed using the Bioconductor packages of R (Gentleman et al., 2004). The data was normalized using the RMA algorithm (Irizarry et al., 2003) with the TAIRG v17 cdf file (http://brainarray.mbni.med.umich.edu).

To equalize background noise, gene expression values less than four were replaced with four (Dekkers et al., 2013). After this transformation, fold changes were calculated comparing each time-point with the control (germinated seeds at the stage of radicle protrusion non-treated with ABA). A gene was considered differentially expressed (the Differentially Expressed Genes: DEGs) if the difference between its mean expression in at least one time-point and the control was statistically significant at $P \leq 0.01$ after application of linear modelling with thresholds for absolute fold change of 2.0 (on a $\log_2$ scale).

The resulting gene set was used for an over-representation analysis (ORA) to recover over-represented biological processes (using Bonferroni-corrected *P*-values at 0.05) based on gene ontologies using Gene Trail (Keller et al., 2008). The term's semantic distance with respect to other semantically close terms ("Dispensability") was calculated using the online tool ReviGO (Supek et al., 2011) and redundant Gene Ontology (GO) terms were removed applying a cut-off of < 0.1 for this "Dispensability" value.

Network construction and analysis

Pearson correlation coefficients between all pairs of DEGs were calculated. A table with correlation coefficient values was exported to

Cytoscape v.2.8.2 (Smoot et al., 2011) and correlation coefficients above a threshold of 0.97 (determined according to Freeman et al., 2007) were used to filter the connections that were used to determine the edges between nodes in the network. The resulting network was displayed with a yGraph Organic layout. The Cytoscape plug-in NetworkAnalyzer (Assenov et al., 2008) was used to compute node degree. The 100 nodes with highest degrees were considered "hubs".

Self-Organizing Maps (SOMs) were calculated using GeneMaths software (version 2.1, Applied Maths BVBA, Sint-Martens-Latem, Belgium) by importing gene expression data and mapping them into six groups (2 x 3 node format) that provided optimal representation of gene expression patterns in a reasonably small number of independent bins.

Mutant analysis

T-DNA insertion lines (Suppl Table 3.1 and Suppl Fig 3.1) for selected genes were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al., 2000). Plants with homozygous T-DNA insertions were selected and confirmed using PCR and grown as described above for wild-type plants. Seeds were bulk-harvested in three replicates of at least two plants. Germinated seeds at the stage of appearance of first root hairs (stage IV, Maia et al., 2011) were phenotyped to evaluate the ability to re-establish DT after treatment with PEG as described by Maia et al. (2011). The evaluated parameters were survival of their primary root, cotyledon survival and seedling survival.

Seeds were also phenotyped for seed dormancy, longevity, and vigour. Seed dormancy was evaluated as the number of Days of Seed Dry Storage required to reach 50% germination (DSDS50) (Alonso-Blanco et al., 2003). Seed longevity was estimated based on an accelerated aging assay (germination percentage after storage for six days at 80% RH and 40˚C in the dark) (Bentsink et al., 2000). Seed vigour was measured as the ability of seeds to germinate at high temperature (at 33˚C) or on NaCl (130 mM). Germination experiments and scoring of germination were performed with the GERMINATOR as described by Joosen et al. (2010) in a fully randomized setup. For each measurement, three replicates of 40 to 60 seeds per seed batch were used.

Results

Re-establishment of DT in germinated *A. thaliana* seeds

Mature *A. thaliana* seeds are desiccation tolerant and imbibition and progression into germination change their status to desiccation sensitive already at the phase of testa rupture (Maia et al., 2011). However, a treatment with ABA fully rescues DT in germinated seeds till the phase of radicle protrusion (Maia et al., 2014). In subsequent developmental stages, DT can no longer be rescued in all organs (Maia et al., 2014).

After 12 h, the treatment with ABA had already led to the re-establishment of DT in 80% of germinated *A. thaliana* seeds at the stage of radicle protrusion (Fig 3.1). After 24 h, all the seeds had re-established DT. To further assess the capacity of survival in the dry state, storability was determined by an accelerated ageing test. Survival rates after the accelerated ageing test were lower for seeds incubated in ABA for 24 h than for seeds incubated in ABA for 72 h (Fig 3.2).

To gain temporal resolution of the changes in gene expression induced by incubation in ABA, gene expression analysis was performed on a time series. Based on the results shown in Fig 3.1, five time-points were chosen for microarray analysis: 0 (control), 2, 12, 24 and 72 h of incubation in ABA.

Principal component analysis was used to compare global changes between the transcriptomes of the different time-points and to evaluate group clustering. One replicate of time point 2 h was a statistically significant outlier (data not shown) and was removed from further analysis. Without this replicate, the first principal component described 61.2% of the variation (Fig 3.3).

In total, 1,177 genes were considered differentially expressed in response to the ABA treatment for at least one time-point as compared to the control. The number of DEGs was the lowest after 2 h of incubation and increased until 24 h, after which no substantial changes occurred until 72 h of incubation (Table 3.1). To verify the accuracy of the microarray data, the expression of 20 genes with different expression patterns was also analysed by qPCR. Both microarray data and qPCR analysis showed comparable trends (Suppl Fig 3.2).

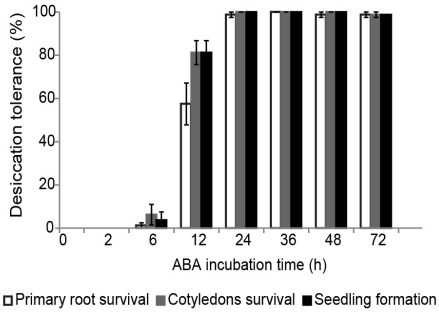

Fig 3.1 *Re-induction of desiccation tolerance in germinated* A. thaliana *seeds at the stage of radicle protrusion during incubation in ABA.* Vertical bars *represent standard error.* Asterisks *indicate significant differences at* $P \leq 0.01$ *comparing each parameter in each time-point and the control.*

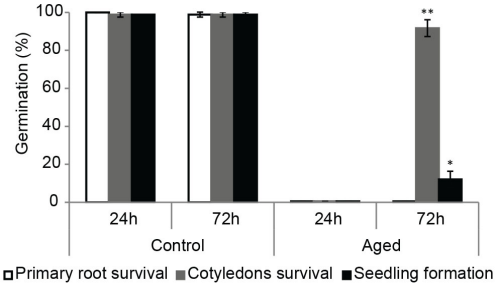

Fig 3.2 *Longevity of germinated* A. thaliana *seeds at the stage of radicle protrusion incubated in ABA for 24 h or 72 h. Longevity was estimated as germination percentage after ageing (24 h of storage at 80% RH and 40°C). Control germinated seeds were incubated in ABA, but not aged.* Asterisks *indicate significant differences at* $P \leq 0.05$ *for one,* $P \leq 0.01$ *for* two asterisks *comparing 24 h and 72 h of incubation in ABA for each parameter in control and aged seeds separated.*

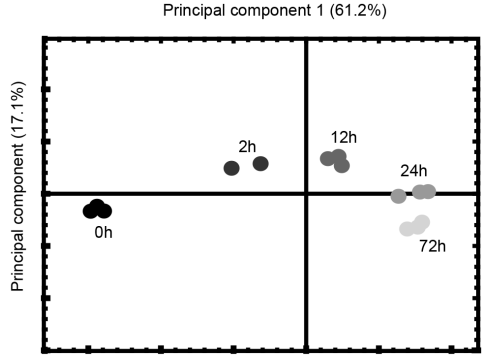

Fig 3.3 *Principal component analysis of microarray data on different time points of ABA incubation.* The different time points *are indicated by* different shades of grey.

Table 3.1 *Distribution of DEGs after statistical analysis comparing each time-point against the control (non treated with ABA). Thresholds were 2.0 for absolute fold change (on a $\log_2$ scale) and 0.01 for* P*-value.*

Time-point (h)	Accumulating	Declining	Total
2	189	111	300
12	297	290	587
24	444	439	883
72	473	394	867

Over-Representation Analysis (ORA) of functional GO categories

ORA was used to get an overview of the enriched functional GO categories comparing the expression data of genes from each time-point relative to the control. Among the genes with accumulating transcript levels, the category lipid storage was highly overrepresented after 2 h of incubation in ABA but, over time, this category became less overrepresented (Fig 3.4). The category dormancy process is over-represented in the genes with accumulating transcript levels only after 24 h of incubation, when DT was fully re-established. Within the genes represented by declining transcript levels, categories related to cell wall (*wax biosynthetic process, cell wall organization or biogenesis, plant-type cell wall organization,* and *syncytium formation*) were over-represented. Genes in these categories are mainly involved in depolymerisation of cell wall components during the beginning of storage mobilization (Sreenivasulu et al., 2008). Categories related to photosynthesis and metabolism (*chlorophyll metabolic process, generation of precursor metabolites and energy,* and *photosynthesis*) were also over-represented within the DEGs represented by declining transcript levels after 12 h and 24 h, when 80% and 100% of the seeds had re-established DT, respectively. Overall, this analysis revealed significant accumulation of transcripts of genes involved in protection, response to stimulus, seed development, and seed dormancy and decline of transcripts of genes related to cell growth, photosynthesis and response to stimulus (Fig 3.4).

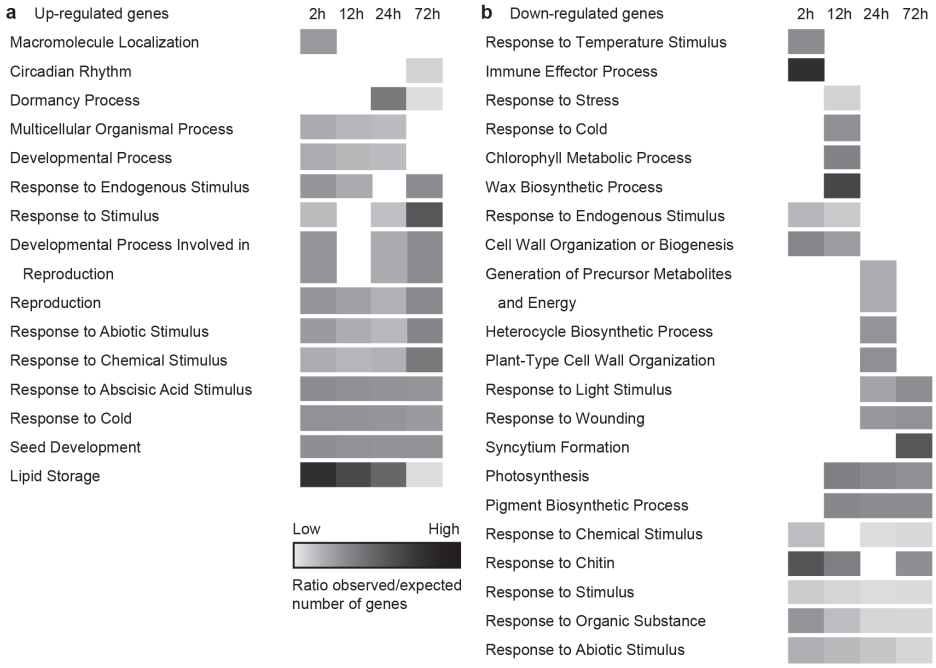

Fig 3.4 *Heat-map of ORA of biological processes on differences between different time-points during incubation of germinated* A. thaliana *seeds in ABA relative to the control (non-treated with ABA). Functional classes were determined using Gene Trail and ReviGO.* P*-values were adjusted for multiple testing by the Bonferroni method applying a cut-off of ≤ 0.05.* a *Results for genes with accumulating transcript levels in each time-point compared to the control.* b *Results for genes with declining transcript levels in each time-point compared to the control.* Shades of grey *indicate the extent (ratio of expected to observed number of genes) to which the category was overrepresented in each comparison.*

Gene co-expression network

Transcriptional studies generate vast amounts of gene expression data. This type of data can be used to build co-expression networks which help to study coordinated gene expression and to identify key genes, functional modules or relations between the network structure, and additional information (Mutwil et al., 2010; Villa-Vialaneix et al., 2013). Using Cytoscape, we constructed a gene co-expression network from the correlation coefficients calculated between all pairs of DEGs. The DEGs are represented as nodes connected by edges that model significant correlation coefficients (Villa-Vialaneix et al., 2013). The

network was built from 1,083 genes/nodes (Suppl Table 3.2) completely connected by 35,296 edges, meaning that any node could be reached from any other node by a path passing along the edges (Villa-Vialaneix et al., 2013) and visualised using an organic layout in Cytoscape (Fig 3.5a).

SOMs can be used to categorize gene expression data into groups that show similar gene expression profiles and thereby may also contain functionally related genes (Törönen et al., 1999). We coloured each node of the co-expression network by the profile group according to the SOM (Fig 3.5b). In this way, two main regions of highly interconnected transcripts were identified: Early Response (ER) and Late Response (LR). The ER region is the most tightly co-regulated one with nodes representing genes with a sharp increase or decrease in expression after 2 h of incubation in ABA. This region concentrates the hubs of the network, meaning that it contains all 100 nodes with the highest degrees (number of nodes directly connected to a given node, ranging from 165 to 209). The LR region contains genes with slow increase or decrease in expression during incubation in ABA.

To assess the cross-link between re-establishment of DT with ABA and seed and stress-related processes, we projected sets of DEGs on the network comparing: dormancy (dormant seeds vs. after-ripened seeds; Cadman et al., 2006, Fig 3.6a); DT acquisition (the cotyledon stage vs. the post mature-green stage of seed development; Le et al., 2010, Fig 3.6b); re-establishment of DT with PEG (germinated seeds at the stage of radicle protrusion before (desiccation sensitive) vs. after (desiccation tolerant) treatment with PEG; Maia et al., 2011, Fig 3.6c); and drought (control plants vs. plants subjected to drought for 10h; Matsui et al., 2008, Fig 3.6d). Genes with increased transcript levels in these comparisons locate mainly in the ER region of the network, while genes with decreased transcript levels locate mainly in the LR region. Moreover, we found an overlap of 16 genes differentially expressed in relation to all these physiological processes (Table 3.2).

After the projection on the network of the DEGs in the re-establishment of DT with PEG, 84% of the nodes were highlighted, with a clear overlap between genes with increasing and decreasing transcript levels in both processes (re-establishment of DT by ABA and PEG) (Fig 3.6c). A comparison between over-represented GO categories in DEGs

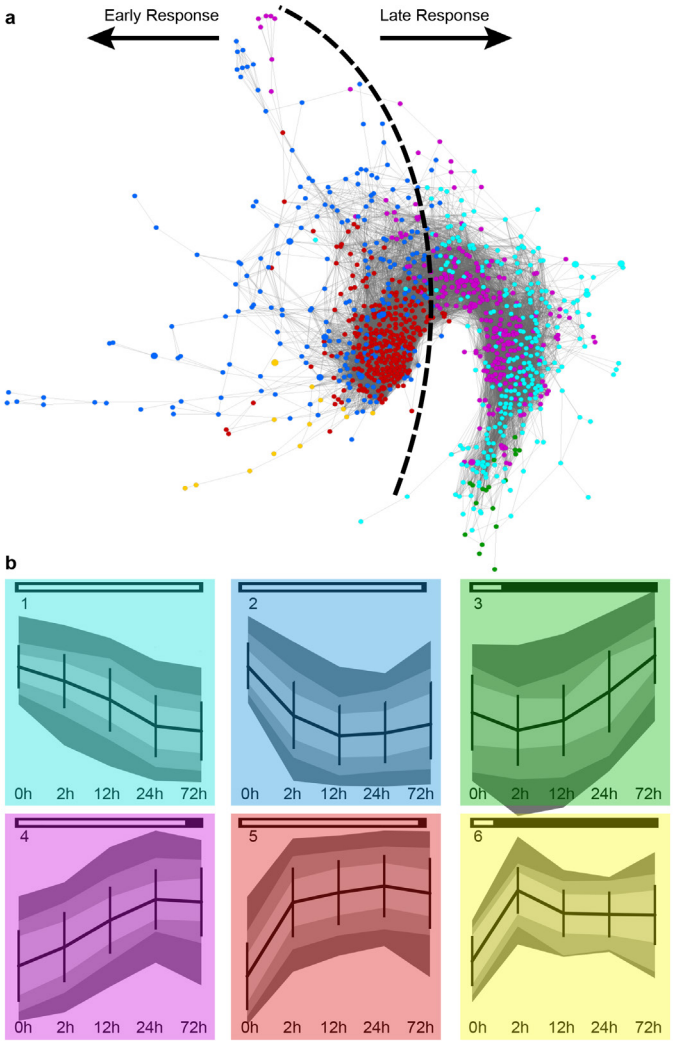

Fig 3.5 *Gene co-expression network* (a) *and SOM* (b) *of transcriptomes of germinated* A. thaliana *seeds incubated in ABA for different intervals. The network is visualized using an yGraph Organic layout in Cytoscape and the temporal analysis of modules was obtained by* coloring *each node by the profile specific for the SOM groups. The* dashed line *and the* arrows *in the network indicate the two regions, namely Early Response and Late Response. For the SOM, the probe sets were grouped into 6 clustered patterns.* Horizontal bars *at the top of each graph represent the number of probe sets belonging to a certain SOM bin. The SOM bins having the highest number of genes have the largest bar.* Vertical bars *represent standard deviation in average expression of the genes in each time point per group. The* three colour shades *in each graph ranging from dark to light correspond to the 98th, 90th and 75th percentiles, respectively.*

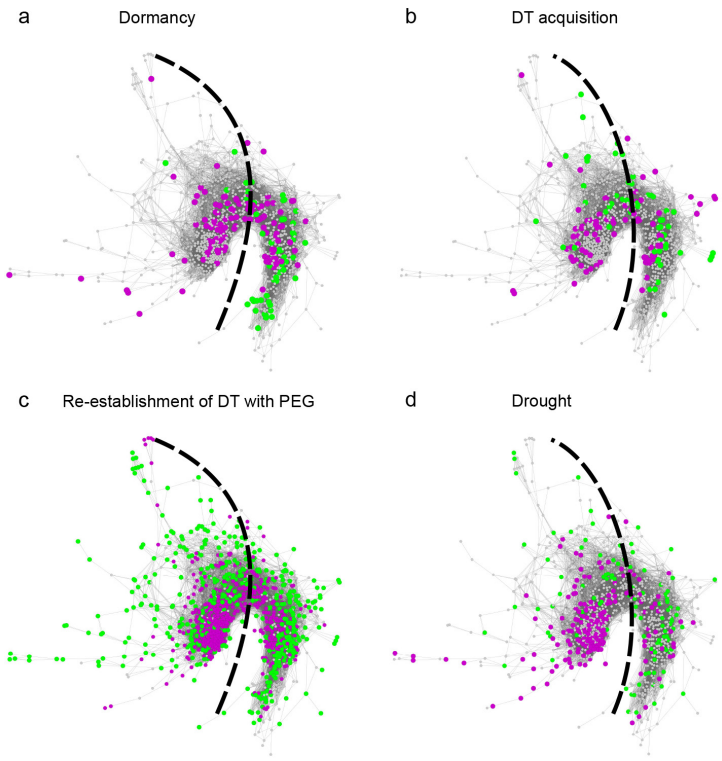

Fig 3.6 *Gene co-expression network of incubation in ABA of germinated* A. thaliana *seeds. Nodes are* colored *according to genes with accumulating (*purple*) or declining (*green*) transcript levels in different datasets comparing:* a *dormancy (dormant seeds vs. after-ripened seeds; Cadman et al., 2006);* b *DT acquisition (cotyledon stage vs. post mature-green stage of seed development; Le et al., 2010);* c *re-establishment of DT with PEG (germinated seeds in the stage of radicle protrusion before (desiccation sensitive) vs. after (desiccation tolerant) treatment with PEG; Maia et al., 2011); and* d *drought (control plants vs. plants dried for 10 h; Matsui et al., 2008). The* dashed line *separates the Early Response (*left*) and the Late Response (*right*) region.*

with accumulating transcript levels in response to incubation in ABA and in PEG revealed a large overlap in categories related to response to stimulus and seed development (Maia et al., 2011). However, the categories *circadian rhythm*, *dormancy process* and *macromolecule localization* were over-represented only in response to incubation in ABA, while categories related to respiration were over-represented only in response to incubation in PEG. ABA regulates the expression of genes related to circadian clock through TFs such as ABI5, AP2/ERF and NAC (Fujita et al., 2011), and the induction and maintenance of dormancy

Table 3.2 *Genes differentially expressed in all the following processes: dormancy (dormant seeds vs. after-ripened seeds; Cadman et al., 2006); DT acquisition (cotyledon stage vs. post mature-green stage of seed development; Le et al., 2010); re-establishment of DT with PEG (germinated seeds in the stage of radicle protrusion before (desiccation sensitive) vs. after (desiccation tolerant) treatment with PEG; Maia et al., 2011); and drought (control plants vs. plants dried for 10 h; Matsui et al., 2008).*

AGI	Annotation
AT1G05340	Unknown protein
AT1G13640	Phosphatidylinositol 3- and 4-kinase family protein
AT1G14530	TOM THREE HOMOLOG 1 (THH1)
AT1G15330	Cystathionine beta-synthase protein
AT1G15740	Leucine-rich repeat family protein
AT1G21680	DPP6 N-terminal domain-like protein
AT1G55530	RING/U-box superfamily protein
AT1G64660	METHIONINE GAMMA-LYASE (MGL)
AT2G16430	PURPLE ACID PHOSPHATASE 10 (PAP10)
AT2G31350	GLYOXALASE 2-5 (GLX2-5)
AT2G33830	Dormancy/auxin-associated family protein
AT3G03310	LECITHIN:CHOLESTEROL ACYLTRANSFERASE 3 (LCAT3)
AT3G20250	PUMILIO 5 (PUM5)
AT3G26580	Tetratricopeptide repeat (TPR)-like superfamily protein
AT4G05390	ROOT FNR 1 (RFNR1)
AT4G29190	OXIDATION-RELATED ZINC FINGER 2 (OZF2)

(Finch-Savage and Leubner-Metzger, 2006). The osmotic stress and a possible hypoxia caused by the incubation in PEG may have induced the expression of genes related to respiration, such as *AT1G19530*, *AT1G33055*, *AT3G10020*, *ADH1*, leading to the over-representation of the categories *anaerobic respiration* and *cellular respiration* (Maia et al., 2011). Considering DEGs with declining transcripts, GO categories related to cell wall, photosynthesis and response to stimulus were over-represented in response to both incubation in ABA and in PEG (Maia et al., 2011). The categories *heterocycle biosynthetic process* and *syncytium* were over-represented only in response to incubation in ABA, while the categories *fatty acid metabolic process* and *sulphur metabolic process* were over-represented only in response to incubation in PEG. The over-representation of these categories is an indication that incubation in either ABA or PEG leads to growth arrest by affecting different genes. Incubation in ABA reduces abundance of transcripts related to expansins (*ATEXPA1*, *ATEXPA8* and *ATEXPA 10*), extensins (*AT2G43150*, *AT3G28550* and *AT3G54580*) and certain photosynthesis-related genes

(*APT3*, *APT5*, *GUN4*, *GUN5*, *HEMA1* and *PORB*) that are not significantly affected by PEG treatment, while PEG reduced the abundance of gene transcripts related to fatty-acid metabolism (*AT5G10160*, *ACP4*, *HCD1*, *OPR1* and *OPR2*) and related to the synthesis of compounds that contain sulphur (*ATGSTU27*, *CYP79B2*, *CYP83B1*, *MAT3* and *MTO3*), such as the amino acid cysteine.

Considering that in plant genomes many TFs are master regulators of signalling and regulatory pathways of stress adaptation and act in an ABA-dependent manner (Lindemose et al., 2013), we searched for the presence of known and predicted TFs as listed in the database of Arabidopsis transcription factors (DATF, http://datf.cbi.pku.edu.cn, Guo et al., 2005) in our network. From the 2,290 TF gene model identifiers available in the DATF, we found 69 in our network (Fig 3.7a). Of these, 46 are located in the ER region and are mainly related to response and tolerance to abiotic stress, such as *Abscisic acid insensitive* (*ABI5*), members of the APETALA 2/ethylene-responsive element binding factor (AP2/ERF) family (*AT5G18450*, *AT5G51190* and *ERF5*), members of the NAM/ATF1/CUC2 (NAC) class (*NAC032*, *NAC048*, *NAC053*, *NAC060*, *NAC083* and *NAC089*) and WRKY TFs (*WRKY 18*, *WRKY29* and *WRKY36*). TFs located in the LR region are mainly related to abiotic stress tolerance, growth regulation and light signalling pathways, such as *ABRE-binding factor 1* (*ABF1*), *enhanced Em levels* (*EEL*), *LATERAL ORGAN BOUNDARY DOMAIN 41* (*LBD41*), *LATE ELONGATED HYPOCOTYL* (*LHY*),

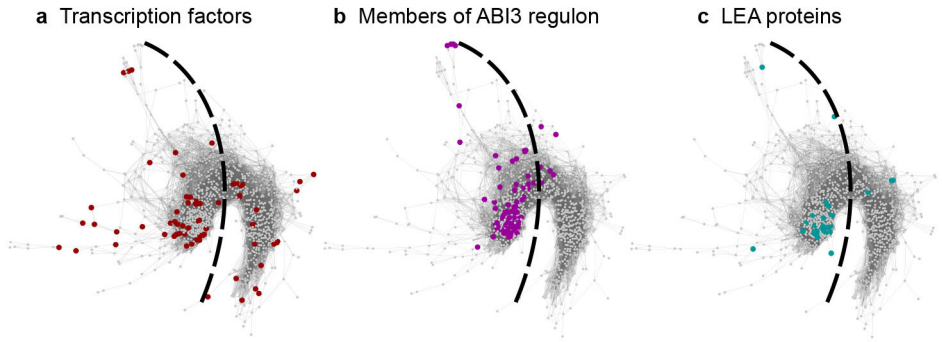

Fig 3.7 *Gene co-expression network of germinated* A. thaliana *seeds incubated in ABA. Nodes are* colored *according to genes encoding* a *transcription factors,* b *members of the ABI3 regulon, and* c *LEA proteins. The* dashed line *separates the Early Response (*left*) and the Late Response (*right*) region of the network.*

PHYTOCHROME-INTERACTING FACTOR-LIKE 2 (*PIL2*), *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*), *PSEUDO-RESPONSE REGULATORS* (*PRR5*), *TIMING OF CAB EXPRESSION 1* (*TOC1*) and *WUSCHEL RELATED HOMEOBOX 12* (*WOX12*).

The TF *ABI3* is a key regulator of seed development and maturation that, together with ABA, plays an essential role in the protection of embryonic structures from desiccation (Mönke et al., 2012). Targets of ABI3 include genes with a protective role in DT, such as LEA genes. We projected the set of target genes from the ABI3 regulon (Mönke et al., 2012) (Fig 3.7b) and the set of genes from the inventory of all LEA proteins identified in the *A. thaliana* genome on our network (Bies-Ethève et al., 2008) (Fig 3.7c). Most of the genes representing the ABI3 regulon and LEA proteins are concentrated in the ER region of the network. Within the hubs identified in the network, genes of the ABI3 regulon and LEA genes are significantly (*P*-value < 0.01) enriched with 44 and 19 genes respectively.

We selected 12 hub genes that are members of the ABI3 regulon, six of which are LEA genes, and investigated their function in the re-establishment of DT and seed-related traits such as dormancy, longevity and vigour (Suppl Table 3.3). These genes were chosen based on indications of their involvement in DT from ongoing studies in our laboratory. The evaluation of seed-related traits is important since the acquisition of DT by seeds requires a series of mechanisms that are also necessary for seed survival in the dry state (Delahaie et al., 2013; Kalemba and Pukacka, 2012) and the disruption of one or more of these mechanisms can impact other aspects important for the completion of germination. We characterized T-DNA insertion lines for each of the aforementioned 12 genes. *ECP63*, *AT2G42560*, *AT3G15670*, *AT3G53040*, *AT4G36600*, and *AT5G44310* are the ones that are both members of the ABI3 regulon and the LEA gene family. The remaining six (*AT1G27990*, *CBSX4*, *RTNLB13*, *AT2G25890*, *AT3G54940*, and *AT4G25580*) are members of the ABI3 regulon that are not LEA genes: *AT1G27990* encodes an unknown protein; *CBSX4* encodes a cystathionine-β-synthase (CBS) domain-containing protein involved in reproduction and is highly expressed in dry seeds (Fang et al., 2011); *RTNLB13* codes for a plant reticulon localized in the tubular endoplasmic reticulum and is predicted to be expressed in seeds (Sparkes et al., 2010); *AT2G25890*

codes for a sugar-regulated oleosin (Huang et al., 2010); *AT3G54940* encodes a papain-like proteinase up-regulated in senescing siliques (Trobacher et al., 2006) and *AT4G25580* encodes a protein related to response to stress and is highly similar to a CAP160 protein linked with cold acclimation (Kaye et al., 1998).

For the phenotypic characterization in relation to re-establishment of DT, germinated seeds in the stage of appearance of first root hairs were used instead of germinated seeds in the stage of radicle protrusion. At the stage of radicle protrusion, germinated seeds of all the lines, including the wild-type, were able to re-establish DT in more than 90% of germinated seeds (data not shown). Therefore, no differences were detected between them. On the other hand, at the stage of appearance of first root hairs, germinated seeds were not able to fully re-establish DT, allowing a clearer separation between wild-type seeds and seeds from T-DNA insertion lines. During evaluation of the survival, frequently, when the primary root fails to survive drying, the hypocotyl remains able to generate lateral roots, allowing normal seedling formation. Therefore, the combined evaluation of root survival, cotyledon survival and seedling formation provides a complete representation of the phenotypic influence of the T-DNA insertion on the re-establishment of DT. Three lines had a significant increase in the ability to re-establish DT compared to the wild-type: *cbsx4*, *at3g53040* and *at4g25580* (Fig 3.8 and Table 3.3). Three different lines had a decrease in seed vigour, with *at1g27990* being affected in high temperature seed vigour, and *at3g54940* and *at5g44310* showing severely reduced germination under salt stress (Table 3.3). The genes disrupted in these six lines had a fast increase in transcript level (expression level increase of more than 4 fold) already after 2 h of incubation in ABA and remained highly expressed but without significant changes until the last time point. None of the lines showed phenotypes for seed dormancy and longevity (Suppl Table 3.3).

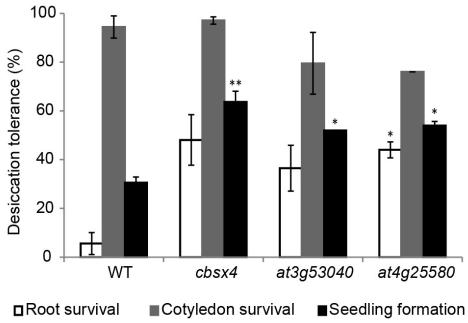

Fig 3.8 *Re-establishment of desiccation tolerance in germinated seeds of wild type (WT) and T-DNA insertion lines scored as survival of primary root (root survival) and cotyledons and seedling formation.* Bars *represent standard error of 3 replicates.* Asterisks *indicate significant differences at* P ≤ *0.05 for one and* P ≤ *0.01 for two asterisks.*

Table 3.3 *Genes chosen for phenotypic characterization with T-DNA insertion lines and their phenotyping results.*

AGI	Annotation	Seed vigor		DT re-induction
		High temperature	Salt	
AT1G27990	Unknown protein	↓↓		
AT1G80090	CBS DOMAIN CONTAINING PROTEIN 4 (CBSX4)			↑↑
AT2G23640	RETICULAN LIKE PROTEIN B13 (RTNLB13)			
AT2G25890	Oleosin family protein			
AT2G36640	EMBRYONIC CELL PROTEIN 63 (ECP63)			
AT2G42560	LEA domain-containing protein			
AT3G15670	LEA family protein			
AT3G53040	LEA protein, putative			↑
AT3G54940	Papain family cysteine protease		↓	
AT4G2558	CAP160 protein			↑
AT4G36600	LEA protein			
AT5G44310	LEA family protein		↓	

Arrows indicate significant increase (↑) or decrease (↓) as compared to the wild type. Single arrows indicate $P \leq 0.05$ and double arrows indicate $P \leq 0.01$

Discussion

Studies focusing on re-establishment of DT often use PEG alone or in combination with low temperature or ABA (Buitink et al., 2003; Maia et al., 2011, 2014; Vieira et al., 2010). In each case, seeds are exposed to a mild osmotic stress that often triggers a series of ABA-related responses (Dalal et al., 2009; Huang et al., 2008; Matsui et al., 2008). In order to evaluate the responses to ABA alone, without additional stressors, we studied germinated *A. thaliana* seeds at the stage of radicle protrusion during incubation in ABA. In this stage, DT can be re-established in all the seeds incubated in ABA already after 24 h of incubation (Fig 3.1), while approximately 85% of seeds incubated in PEG for 24 h had re-established DT (Maia et al., 2011). DT re-established in all the seeds by incubation in PEG is only obtained after 72 h of incubation (Maia et al., 2011).

In general, osmotic stresses trigger a series of physiological responses in ABA-dependent and ABA-independent manners with the existence of a cross-talk between them (Buitink et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). A comparison between over-represented GO categories in DEGs with accumulating transcripts in response to incubation in ABA and in PEG reinforced the idea that these treatments lead to the re-establishment of DT through partially overlapping mechanisms.

A closer look at the main processes that occur during incubation in ABA revealed enrichment of different GO categories of biological processes (Fig 3.4), attesting the growth arrest and the partial return to a quiescent stage similar to the dry seed (Buitink et al., 2006; Maia et al., 2011).

Lipid storage was the most over-represented GO category within the genes with accumulating transcript levels and the genes inside this category are oleosins and oleosin family proteins. Oleosins are typical for the later stages of seed development and, in desiccation tolerant seeds, they prevent lipid bodies from coalescing on dehydration and prevent the disruption of cellular structures during rehydration (Leprince et al., 1998; Pammenter and Berjak, 1999).

Despite the fact that all the germinated seeds incubated in ABA

had re-established DT after 24 h, the incubation was maintained for a total of 72 h, which improves longevity compared with incubation for 24 h (Fig 3.2). Also during development, acquisition of DT precedes acquisition of longevity, likely to ensure timely response of stress-related genes (such as LEAs, HSPs and oxidative stress-related genes) that also improve seed storability (Verdier et al., 2013).

Transcriptomic studies generate large datasets. Visualization and analysis of this kind of data as networks is an important approach to explore a wide variety of biological relations (Dekkers et al., 2013; Freeman et al., 2007). To construct a network, the similarity between individual expression profiles may be determined and used as edges that connect nodes, or genes (Freeman et al., 2007). Once the network is given, an analysis of its structure can indicate key genes, functional modules or relations between the network structure and additional information (Villa-Vialaneix et al., 2013). Several centrality measures or topological indexes have been used to analyse networks. The degree centrality or just degree is thought to be simplest (Bass et al., 2013; Piraveenan et al., 2013; Scardoni et al., 2009). It refers to the number of links a given node has with other nodes and allows an immediate evaluation of the relevance of the node to the network (Piraveenan et al., 2013; Scardoni et al., 2009). Nodes with high degrees are called hubs and are thought to play important roles in organizing the behaviour of the network (Bass et al., 2013; Dong and Horvath, 2007; Villa-Vialaneix et al., 2013).

The ER region of the network contains genes related mainly to wax biosynthetic processes, lipid storage, seed development and response to abscisic acid stimulus. Most of the genes with accumulating transcript levels in relation with dormancy, DT acquisition, re-establishment of DT with PEG, and drought also locate in this region (Fig 3.6). Also, all the hubs and most of the members of the ABI3 regulon, LEA proteins and TFs found in the network are in this region (Fig 3.7). ABA is a key component of responses to abiotic stresses and regulation of seed dormancy and germination. The responses to abiotic stresses elicited by ABA include minimization of water transpiration (for example, by the accumulation of cuticular wax), and synthesis of protectants (such as LEA proteins) and antioxidants (Dalal et al., 2009; Seo et al., 2011). During seed

development and germination, several genes under the control of ABA are related to the acquisition and loss of dormancy and DT (Buitink et al., 2006; Maia et al., 2011; Terrasson et al., 2013; Toorop et al., 2005). Moreover, ABA-responsive gene expression is directly regulated by TFs (Fujita et al., 2011). The TFs in the ER region of the network, such as ABI5, AP2/ERF TFs and NAC TFs, are known to be related to response and tolerance to abiotic stress possibly by a rapid amplification and broadening of signal responses (Fujita et al., 2011). Taken together, these observations reinforce the relation between early stress response and seed development and the view that early responsive genes may provide initial protection and amplification of signals (Buitink et al., 2006).

The LR region contains genes related mainly to response to abiotic stimulus (especially light stimulus), and aromatic amino acid family metabolic processes. The GO category *aromatic amino acid family metabolic processes* refers to chemical reactions and pathways involving amino acids with an aromatic ring, such as phenylalanine, tyrosine, and tryptophan, which could be involved in protein stability (Carbon et al., 2009; Khuri et al., 2001). TFs located in this region are related to abiotic stress tolerance, growth regulation and light signalling pathways and might influence the plant's ability to adapt to daily changes in water status in a coordinated action with the circadian clock (Alabadí et al., 2001; Fujita et al., 2011). These results are in agreement with the hypothesis that genes that are responding later may be involved in adaptation to stress conditions (Buitink et al., 2006).

We considered the 100 nodes with highest degrees as hubs. They are all concentrated in the ER region and most of them follow the expression pattern corresponding to the 5th clustered pattern of the SOM analysis (fast increase in expression in the first 2 h of incubation in ABA followed by certain stabilization in the following hours).

Three T-DNA insertion lines showed a higher ability to re-establish DT compared to wild-type, suggesting the existence of de-repression mechanisms and redundancy between highly similar genes. These lines are *cbsx4*, *at3g53040* and *at4g25580*. *CBSX4* is one of the two *A. thaliana* homologous genes (the other is *AT1G15330*) that encode CBS domain-containing proteins that belongs to the PV42 class of

gamma subunits of SnRK1 (Fang et al., 2011). CBS domain-containing proteins have been found to act in a variety of biological processes, such as metabolic enzymes, transcriptional regulators, ion channels, and transporters (Fang et al., 2011; Rosnoblet et al., 2007). *A. thaliana* mutants in *HISTONE ACETYLTRANSFERASE1* (*HAC1*) had reduced expression of both *AT1G15330* and *CBSX4*, suggesting a role of these genes in sugar sensing and fertility (Heisel et al., 2013). Phenotyping a T-DNA insertion line for *AT1G15330* did not show any phenotypes (data not shown). The observed phenotype of higher ability to re-establish DT compared to wild-type seeds could be due to compensatory effects of one homologous gene over the other. Interestingly, *cbsx4* had an increased expression of the mutated gene compared to the wild-type in a RT-PCR experiment. We speculate that the amplification of this gene generates a product with a non-functional sub-unit, as the presence and location of the insertion in the last exon of the gene was confirmed.

AT3G53040 codes for a cytosolic LEA domain-containing protein that shares typical features of the LEAM protein class A α-helix motifs, being classified as LEA_4 family (Candat et al., 2014). Proteins of this family are likely to interact and protect various cellular membranes during dehydration (Candat et al., 2014). In *A. thaliana* seedlings, the expression of *AT3G53040* was shown to respond to ABA, but without inducible expression to stress conditions (Bies-Ethève et al., 2008; Huang and Wu, 2006). *AT3G53040* and *AT2G36640* are very similar to each other in sequence and expression pattern and are considered to form a pair derived from a whole genome duplication (Bies-Ethève et al., 2008; Hundertmark and Hincha, 2008). It is expected that duplicated genes have redundant functions and the higher ability to re-establish DT observed in *at3g53040* compared to wild-type could be caused by this redundancy and compensatory effects. It is likely that the phenotyping of a T-DNA insertion line of *AT2G36640* did not result in differences compared to the wild-type (data not shown) due to this redundancy.

AT4G25580 codes for a stress-responsive protein-related with weak similarity (less than 20% identity) with LEA group 2 (Bies-Ethève et al., 2008) and high similarity to a CAP160 protein (Mönke et al., 2012) and to a CDeT11-24 from *Craterostigma plantagineum* (Röhrig et al., 2006). In spinach, CAP160 proteins are predominantly cytosolic,

induced by drought stress and related to stabilization of membranes, ribosomes and cytoskeletal elements (Kaye et al., 1998). CDeT11-24 is thought to contribute to the plant's DT possibly by interacting with other proteins such as dehydrins (Röhrig et al., 2006). However, *at4g25580* seeds had an increased ability to re-establish DT compared to wild-type seeds, suggesting the operation of derepression mechanisms.

None of the T-DNA lines analysed showed phenotypes for seed dormancy or longevity. Only one of the analysed genes (*AT3G53040*) was reported to be differentially expressed comparing dormant and after-ripened states (Bassel et al., 2011; Cadman et al., 2006). Considering the increase in longevity between 24 h and 72 h of incubation in ABA and the location in the network (ER region) of the genes chosen for phenotypic characterization, it is conceivable that the disruption of single genes would not influence longevity. Besides, as only three of the T-DNA lines had the vigour affected, we believe that seed development was not significantly affected by the disruption of the single genes.

Taken together, these results confirm that the analysis of co-expression network structures can bring insights to biological processes. We showed that the re-establishment of DT in germinated *A. thaliana* seeds can be divided in two phases. In the first phase, a series of stress-responsive genes that are also related to seed development as well as other biological processes (such as dormancy, acquisition of DT, drought and the circadian clock) is induced, promoting amplification of signals, growth arrest and protection mechanisms (such as LEA proteins). In the second phase, photosynthesis and primary metabolism are strongly inhibited and another set of stress-responsive genes promotes adaptation to stress conditions that also contribute to seed survival in the dry state, improving longevity. Moreover, we suggest that redundancy and compensatory mechanisms may be operating when genes important for DT are disrupted.

Acknowledgements

This work was supported by the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Brazil).

Author Contribution Statement

MCDC designed research, conducted experiments, analysed data and wrote the manuscript. KR analysed data and wrote the manuscript. HN designed scripts for microarray analysis. FY conducted experiments. WL, JB and HWMH conceived and designed research, analysed data and wrote the manuscript. All authors read and approved the manuscript.

Supplementary Data

Supplementary Data can be downloaded by scanning, from the online version of this article (Costa et al., 2015) or from: http://www.wageningenseedlab.nl/thesis/mcdcosta/SI/chapter3

Suppl Fig 3.1 Schematic illustration and relative abundance of transcripts of mutated genes in *cbsx4*, *at3g53040* and *at4g25580*. **a** Mapped T-DNA insertion sites (*triangles*) are indicated on the top of the genomic structure. *Black boxes* on the *black solid line* indicate the exons, *grey boxes* indicate 3'and 5' untranslated regions and *arrows* indicate location of qPCR primers (fwd: forward; rev: reverse). **b** Normalized relative expression levels of transcripts of mutated genes in *cbsx4*, *at3g53040* and *at4g25580* compared to wild-type (WT) calculated with the qBase software (Hellemans et al., 2007). *Asterisks* indicate significant differences at $P \leq 0.05$ for one, $P \leq 0.01$ for two asterisks.

Suppl Fig 3.2 Temporal expression profiles of 20 genes measured by qPCR and microarray after incubation of germinated seeds in the stage of radicle protrusion for 0 h, 2 h and 24 h. *Blue lines* indicate expression levels measured by qPCR and *red lines* indicate expression levels measured by microarray.

Suppl Table 3.1 T-DNA knock-out lines used for this study.

Suppl Table 3.2 List of 1,083 probe sets used to build the network.

Suppl Table 3.3 Phenotyping results of T-DNA knock-out lines. Germinated seed in the stage of appearance of first root hairs (stage IV, Maia et al., 2011) were phenotyped to evaluate the ability to re-establish desiccation tolerance by PEG treatment and three parameters were evaluated: survival of primary root (root survival), cotyledons survival and seedling survival. Seed were also phenotyped in relation to seed vigour (ability to germinate at high temperature (at 33˚C) or on salt (130 mM NaCl), dormancy (number of Days of Seed Dry Storage required to reach 50% germination, DSDS50), and longevity (germination percentage after storage for six days at 80% RH and 40˚C in the dark). Significant differences at $P \leq 0.05$ are in bold.

Chapter
Four

A linkage between drought tolerance and desiccation tolerance in rice

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*These authors contributed equally to this work

In preparation

Abstract

Rice is the most important staple food in the world. Most of the world's rice production depends on irrigation and it has been estimated that rice uses 2-3 times more water per hectare than other irrigated crops. In a future with predicted lower availability of fresh water, rice production may be dramatically affected, resulting in large yield losses. Efforts to increase rice drought tolerance without reducing yield have already identified cultivars with lower drought sensitivity. More advances could be achieved by combining drought and desiccation tolerance (DT) studies. Hence, we searched for a linkage between drought tolerance in adult rice plants and DT in germinated rice seeds. To do so, we used eight rice cultivars differing in drought tolerance. Dular, a drought tolerant cultivar, lost DT in a later developmental stage than the other cultivars, possibly as an effect of a late start of cell division. The results suggest that the intrinsic mechanisms of drought tolerance in adult plants are a part of the mechanisms used by seeds to tolerate desiccation. Further studies are necessary to uncover the molecular nature of these mechanisms.

Keywords

Cell division; Drought stress; Stress tolerance

Introduction

During its 8000-9000 years of cultivation by humans, rice became the main source of daily calories for millions of people, especially in Asian countries (Elert, 2014). Rice is mainly cultivated in paddies where irrigation increases yields by 70%, as compared to rain-dependent fields (Lenka et al., 2011). However, predictions that by 2070 irrigation water availability will be extremely limited increase the need to improve agricultural productivity by other means (Elliott et al., 2014). Therefore, in a context of predicted drier climatic conditions, efforts to increase rice drought tolerance without compromising productivity are crucial.

Drought in agriculture occurs when the amount of water available to plants is less than what these plants require to sustain maximum growth and productivity (Moore et al., 2008). Plants can deal with drought by escaping, avoiding or tolerating the stress (Dekkers et al., 2015; Verslues and Juenger, 2011). Drought tolerance has been defined as the capacity of plants to tolerate moderate dehydration, down to ~0.3 g H_2O per gram dry weight (Dekkers et al., 2015). Responses of plants to resist drought involve adaptations to prevent water loss while growing under reduced water potential (Moore et al., 2008; Verslues and Juenger, 2011). Various cultivars of rice and other crop species prevent damage caused by water loss by improving water use efficiency and osmotic adjustment (Moore et al., 2008). When drought becomes more severe, drought tolerant cultivars activate cell wall adjustments, reduce growth, accumulate antioxidants and down-regulate photosynthesis (Verslues and Juenger, 2011). If drought persists, only desiccation tolerant plants will survive.

Desiccation tolerance (DT) denotes the capacity to tolerate severe dehydration, below 0.1 g H_2O per gram dry weight (Dekkers et al., 2015). It involves the activation of a series of mechanisms to ensure cellular integrity and repair, such as vacuole filling; accumulation of heat sock proteins and antioxidants; and cell wall folding (Dekkers et al., 2015; Verslues and Juenger, 2011). Drought tolerance and DT share a wide range of mechanisms. These include accumulation of sugars and late embryogenesis abundant proteins; abscisic acid (ABA) mediated

responses and expression of DRE/CRT (dehydration-responsive element/C-repeat), DREB/CBF (DRE-binding protein/C-repeat binding factor) and Myb-related transcription factors (reviewed by Le and McQueen-Mason, 2006).

Seeds of more than 90% of Angiosperm species are desiccation tolerant (Royal Botanic Gardens Kew, 2008), but during imbibition and germination, DT is progressively lost (reviewed by Dekkers et al., 2015). Several studies demonstrated the existence of a developmental window after radicle protrusion during which lost DT can be re-established by treatment with an osmoticum (polyethylene glycol, PEG) and/or ABA (reviewed by Dekkers et al., 2015).

In this study, we hypothesize that the intrinsic drought tolerance of adult plants is part of the DT mechanisms of seeds. Therefore, we searched for a relation between drought tolerance in adult plants and DT in germinated seeds using rice cultivars differing in drought tolerance as a model system. In the same developmental stage, drought tolerant cultivars were able to re-establish DT to higher percentages than drought sensitive ones.

Materials and methods

Plant material

Seven *Indica* rice cultivars with contrasting drought tolerance were used for this study; drought tolerant: 'Apo', 'Salumpikit' and 'IR-45'; drought sensitive: 'IR-20', 'IR-36', 'IR-64' and 'IR-72'. Also 'Dular', an *Aus* rice cultivar was used. Seeds of these cultivars were provided by the International Rice Research Institute, Los Baños, Laguna, Philippines. For optimization of the protocol to re-establish DT (Suppl Fig 4.1), to determine the developmental stage when DT was lost and to measure the mitotic activity of germinating seeds, a commercial *Indica* cultivar was used.

Germination assays

Prior to imbibition, seeds were surface sterilized by briefly washing with 70% ethanol, soaking in 2% sodium hypochlorite for 30 min and then washing thoroughly with demineralized water.

Germination assays were carried out in plastic trays (15 x 21 cm, DBP Plastics, http://www.dbp.be) with 2 layers of blue filter paper (Blue Blotter Paper, Anchor Paper Company, http://seedpaper.com) and 80 ml of distilled water. The plastic trays were kept in germination cabinets with constant white light at 28°C.

Loss and re-establishment of desiccation tolerance

To assess the loss and re-establishment of DT, seeds were grouped as follows: (stage I) 8 h after imbibition; (stage II) seeds at coleoptile protrusion; (stage III) seeds at radicle protrusion; and (stage IV) seeds showing coleoptile and/or radicle longer than 2 mm (Fig 4.1). Three replicates of at least 15 seeds for each stage were either fast-dried directly or after 3 days of incubation in PEG 8000 (-3.0 MPa) at 20˚C. Fast drying was achieved by placing the seeds for three days at 20˚C in the dark in a closed chamber at 44% relative humidity (RH) with forced airflow, resulting in final water contents as low as 0.05 g H_2O g^{-1} dry weight in embryos and 0.12 g H_2O g^{-1} dry weight in the endosperm (Suppl Fig 4.2). Water contents were assessed gravimetrically for triplicate samples of 15 excised embryos or endosperms (without the embryo), by determination of the fresh weight and, subsequently, dry weight after 20 h at 130°C (Chen, 2003). Incubation in PEG was done in the dark at 20˚C on one layer of filter paper in 9 cm Petri dishes containing 6 ml of PEG solution with an osmotic potential of -3.0 MPa. After 3 days of PEG incubation followed by thorough rinsing with distilled water to remove residual PEG, the seeds were transferred to new Petri dishes containing one dry sheet of germination paper and fast-dried. During the drying step, samples were taken at intervals to measure water content (Suppl Fig 4.2). After drying, germinated seeds were pre-humidified in humid air (100% RH) for 24 h at 22˚C and then rehydrated in H_2O at the same conditions as used for germination. Germinated seeds that resumed

growth (growth of leaves and secondary root system) were considered desiccation tolerant.

Fig 4.1 *Rice seeds at different developmental stages during and after visible germination. I - 8 h after imbibition; II - seeds at coleoptile protrusion; III - seeds at radicle protrusion; and IV - seeds showing coleoptile and/or radicle longer than 2 mm.*

Mitotic activity

The mitotic activity was calculated in 1,000 cells from 4-7 sections of shoot and root meristems by scoring cells showing the mitotic figures of prophase, metaphase, anaphase and telophase (Fig 4.2). Sections were fixated in a 3:1 96% ethanol : acetic acid mixture for 1 h and stained in 0.2 µg/ml DAPI in pH 5.0 phosphate buffer (10 mM) for 48 h. The stained sections were examined under a Nikon Optiphot-2 microscope. A Nikon super high-pressure mercury power supply (model HB-10101AF) and a DM400 filter cube WU (excitation filter: 365 nm, barrier filter: 420 nm) were used for the fluorescence imaging. Images were taken using a Zeiss LSM 510-META Confocal laser scanning microscope with a EC Plan-Neofluar 40x/1.3 Oil DIC objective lens.

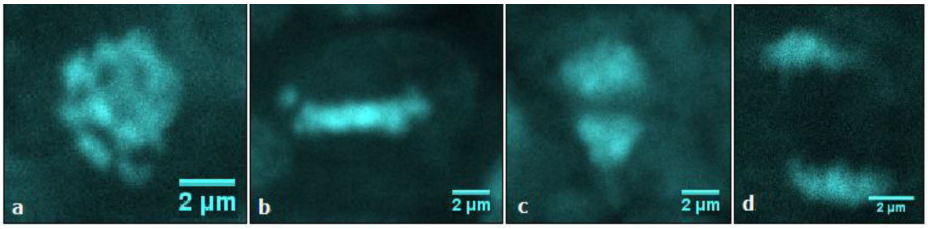


Fig 4.2 *Examples of nuclei of rice meristem cells showing the mitotic figures* a *prophase,* b *metaphase,* c *anaphase, and* d *telophase*

Results and discussion

Rice seeds acquire both the ability to germinate and to tolerate drying to approximately 10% moisture content almost synchronously during seed development, before the end of the seed-filling phase (Ellis and Hong, 1994). During seed maturation, rice seeds continue to develop DT and become tolerant to drying to approximately 5% moisture content (Ellis and Hong, 1994). Later, during imbibition and progression to germination, they become desiccation sensitive. In order to determine the moment of loss of DT, four developmental stages were defined (Fig 4.1). At stage II, DT is already completely lost in seeds of the commercial Indica cultivar used in this study (Fig 4.3).

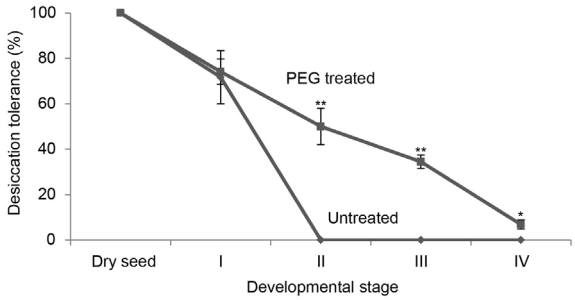


Fig 4.3 *Loss and re-establishment of desiccation tolerance in rice (commercial* Indica *cultivar). DT was determined after drying the seeds with or without previous PEG treatment (-3.0 MPa), followed by pre-humidification and rehydration. Growth resumption was scored 6 days after rehydration. Each data point is the average of three replicates of at least 15 seeds.* Bars *represent standard error.* Asterisks *indicate significant (Student's t test) differences at* $P \leq 0.05$ *for one and* $P \leq 0.01$ *for two asterisks comparing untreated and PEG treated seeds for each stage.*

To determine the best developmental stage suitable to study the re-establishment of DT, seeds in each stage were treated with PEG (-3.0 MPa) solution. For stages II to IV, DT could be re-established to a certain extent, but none of them reached 100% (Fig 4.3). Survival of seeds was dependent on the appearance of secondary roots, as the radicle remained desiccation sensitive.

Due to the highest contrast between PEG treated and untreated samples, seeds in stage II were chosen for evaluation of the ability to re-establish DT in eight different rice cultivars. However, the exact moment

of complete loss of DT in untreated seeds varied slightly between cultivars (Fig 4.4). For most of the cultivars used in this study, 10% or less of the seeds were desiccation tolerant when the coleoptile protruded (stage II). Seeds of Salumpikit and IR-20 were not able to significantly re-acquire DT upon the PEG treatment. Salumpikit is regarded as drought tolerant and well adapted to upland conditions, while IR-20 is a lowland paddy-rice sensitive to drought stress (Price et al., 1997).

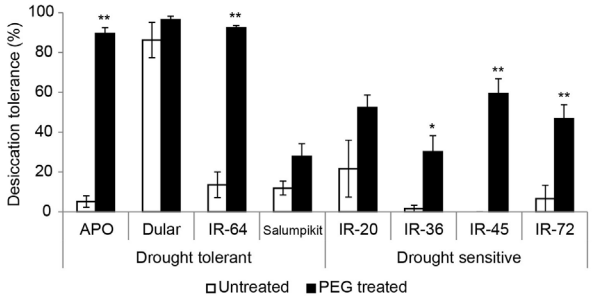


Fig 4.4 *Re-establishment of DT in rice seeds from cultivars differing in drought tolerance. Germinated seeds at coleoptile protrusion (stage II) were used. DT was determined after drying the seeds with or without previous PEG treatment (-3.0 MPa), followed by pre-humidification and rehydration. Growth resumption was scored 6 days after rehydration. Each data point is the average of three replicates of at least 15 seeds.* Bars *represent standard error.* Asterisks *indicate significant (Student's t test) differences at* $P \leq 0.05$ *for one and* $P \leq 0.01$ *for two asterisks comparing untreated and PEG treated seeds for each cultivar.*

In seeds of the cultivar Dular, the PEG treatment did not improve DT significantly because a very high percentage of untreated seeds (around 85%) was still desiccation tolerant at stage II. These seeds became sensitive before the next stage (radicle protrusion, stage III, Fig 4.5). Dular is classified as an *Aus* type and is expected to display greater drought resistance compared with other types of rice (Henry et al., 2011). Interestingly, Dular seeds grew slower than most of the other selected cultivars, taking 2 to 12 h more to reach stage II (Fig 4.6). Constitutive expression of rice genes that cause growth reduction was shown to enhance abiotic stress tolerance in transgenic plants (Tang et al., 2012). In leaves, drought reduces both cell number and size by reversibly inhibiting the transition to the DNA replication phase (S-phase)

(reviewed by Skirycz and Inzé, 2010). Moreover, DNA content has been related to stress tolerance, with higher DNA content increasing stress sensitivity (Faria et al., 2005). In the commercial rice variety, seeds at stage II showed mitotic activity, contrasting with seeds in stage I (Table 4.1). We speculate that germinated seeds of the cultivar Dular take advantage of a selection of drought mechanisms present in adult plants, such as lower DNA content due to a later start of the cell cycle, to extend the period of time when they are desiccation tolerant. As a result, the germinated seeds would be drought resistant for a longer time during the crucial moment of seedling establishment.

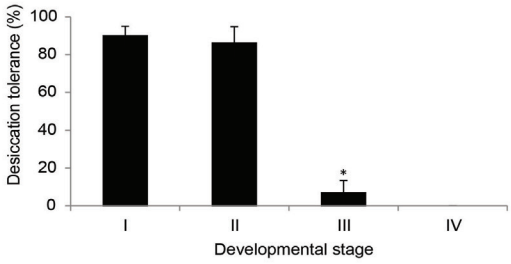


Fig 4.5 *DT in Dullar germinated seeds. DT was determined after drying the seeds, followed by pre-humidification and rehydration. Growth resumption was scored 6 days after rehydration. Each data point is the average of three replicates of at least 15 seeds.* Bars *represent standard error. The* asterisk *indicates significant difference at* P ≤ *0.01 (Student's t test) comparing each developmental stage with the previous stage.*

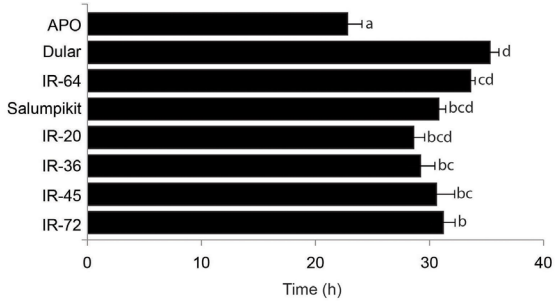


Fig 4.6 *Average hours of imbibition required for seeds to reach developmental stage II. Each data point is the average of 10 individual seeds.* Bars *represent standard error.* Letters *indicate significant difference at* P ≤ *0.05 after one-way ANOVA with Tukey post-hoc test.*

Table 4.1 *Cell mitotic index in root and shoot apical meristems of germinated rice seeds (commercial* Indica *cultivar) in the different developmental stages. The mitotic index was calculated in 1,000 cells from 4-7 sections of shoot and root meristems.*

Stage	Root apical meristem	Shoot apical meristem
I	0%	0%
II	0%	2%
III	0%	1%
IV	3%	0%

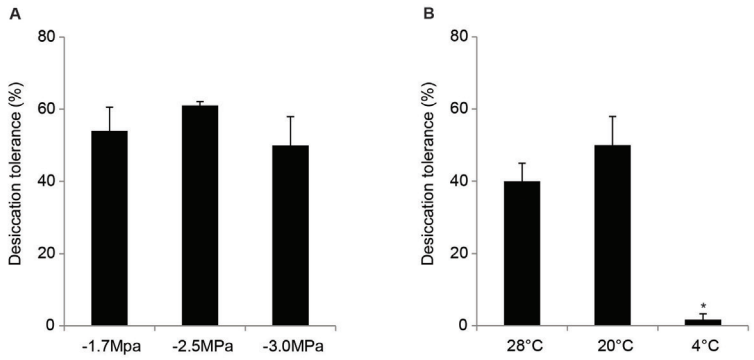
The development of new stress-tolerant crop varieties to face a drier future requires further understanding of plant responses to stress conditions. Part of this understanding should aim at overcoming the fact that increased drought stress tolerance comes at the expense of biomass accumulation (Moore et al., 2008; Skirycz and Inzé, 2010). In the case of rice, Dular has been reported to show lower reduction in yield by drought stress than other rice cultivars (Skirycz and Inzé, 2010; Henry et al., 2011). Therefore, germinated Dular seeds seem to be a very useful model system for understanding the mechanisms underlying growth under stress conditions and the relation between vegetative drought tolerance and seed DT.

Our results support the idea that the intrinsic mechanisms of drought tolerance in adult plants are a part of the mechanisms used by seeds to tolerate desiccation. More studies are necessary to unveil the molecular nature of these mechanisms.

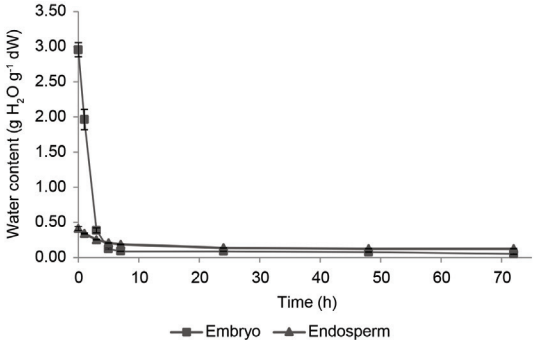
Acknowledgements

This work was supported by the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Brazil). The authors thank Beatriz Andreo Jimenez for assisting with the setup of the germination experiments.

Supplementary Data



Suppl Fig 4.1 Percentage of re-establishment of desiccation tolerance of germinated seeds of a commercial *Indica* rice cultivar in stage II after 72 h of incubation in PEG solutions. **A** PEG solutions with different osmotic potentials at 20˚C. **B** -3.0 MPa PEG solution at different temperatures. Each data point is the average of three replicates of at least 15 seeds. *Bars* represent standard error. *Asterisks* indicate significant differences at $P \leq 0.05$.



Suppl Fig 4.2 Decrease in water content in embryo and endosperm of germinated seeds of a commercial *Indica* rice cultivar in stage II that were dried directly. Each data point represents embryo or endosperm of 15 seeds. *Bars* represent standard error.

Chapter
Five

Longevity of germinated *Sesbania virgata* seeds

Maria Cecília D. Costa; José Marcio Rocha Faria; Anderson Cleiton José; Wilco Ligterink; Henk W. M. Hilhorst

In preparation

Abstract

Seed desiccation tolerance (DT) and longevity are necessary for better dissemination of plant species and establishment of soil seed banks. Both DT and longevity are acquired by orthodox seeds during the maturation phase of development and lost upon germination. DT can be re-induced in germinated seeds by an osmotic and/ or abscisic acid treatment. However, there is no information on how these treatments affect seed longevity. We used germinated *Sesbania virgata* seeds as a model system to investigate the effects of an osmotic treatment to re-establish DT on seed longevity. *S. virgata* is a tree species used on a large scale in agroforestry and ecological restoration. Its seeds are orthodox and lose DT slowly with progression of radicle growth. The radicle is more sensitive organ to desiccation than the cotyledons and the ability to produce lateral roots is key for seedling survival. Germinated *S. virgata* seeds with 1 mm protruded radicle tolerate desiccation and storage for up to 3 months without significant losses in viability. An osmotic treatment can improve DT in these seeds, but not longevity. Consequently, germinated *S. virgata* seeds are a good model to study DT uncoupled from longevity. Further studies are necessary to unveil the molecular mechanisms involved in both DT and longevity.

Key-words

Desiccation tolerance; Storage; Germination; Osmotic stress

Introduction

Species from the genus *Sesbania* (Fabaceae) are distributed mainly in the African and American continents. Due to their fast growth, easy propagation, high biomass production, and potential to form symbiosis with nitrogen-fixing bacteria, they are used on a large scale in agroforestry and for ecological restorations (Florentino et al., 2009; Kwesiga et al., 1999; Ståhl et al., 2005; Zanandrea et al., 2009). One species of this genus commonly used in agroforestry in Brazil is *Sesbania virgata*, a fast-growing pioneer species that tolerates long periods of flooding and has a highly branched root system that protects the soil against erosion (Florentino et al., 2009; Zanandrea et al., 2009).

Seeds of *S. virgata* are orthodox, meaning that they are able to tolerate desiccation and survive in the dehydrated state for long periods (Pammenter and Berjak, 1999). In orthodox seeds, desiccation tolerance (DT) and longevity are considered necessary for the completion of their life cycle, permitting the plant to store seeds, and ensure better dissemination of the species (Ramanjulu and Bartels, 2002).

During the maturation phase, orthodox seeds acquire DT and longevity, enter a dormant or quiescent state and can remain apparently inactive for very long periods (Ooms et al., 1993; Toldi et al., 2009). During germination, upon imbibition, DT remains for some time but then starts to be lost when DNA synthesis and (somewhat later) cell division resume (Faria et al., 2005). After the loss of DT, the existence of a small developmental window during which DT can be re-established by treatment with an osmoticum and/or the plant hormone abscisic acid (ABA) was demonstrated in a number of species, including *Cucumis sativus*, *Impatiens walleriana* (Bruggink and van der Toorn, 1995), *Medicago truncatula* (Buitink et al., 2003, 2006) and *Arabidopsis thaliana* (Maia et al., 2011, 2014). When DT is fully rescued, seeds seem to be in a stage resembling the developmental stage that they were in prior to germination (Buitink et al., 2006; Maia et al., 2011).

The ability of germinated seeds to re-acquire DT is thought to optimize successful seedling establishment under unpredictable environmental conditions (Dekkers et al., 2015). This ability, in

conjunction with longevity, could represent an ecologically important stress tolerance mechanism that allows germinating/germinated seeds to remain viable in the dry state for a certain time. In the last few years, several studies have been carried out focusing on the acquisition, loss and re-induction of DT in seeds of model species (Dekkers et al., 2015). However, some diversity in stress-tolerance mechanisms is expected, raising the expectations that valuable information can be generated using non-model species that have to cope with such stresses in their natural environments. In their natural habitat, *S. virgata* seedlings are subjected to irregular precipitation patterns at the start of the wet season. Despite the numerous studies on the re-induction of DT in germinated seeds, no information is available concerning the effects of treatments to re-induce DT on longevity. In the present study, we investigated the longevity of germinated *S. virgata* seeds. We show that germinated *S. virgata* seeds with 1 mm protruded radicle can be dried back and be stored, and that an osmotic treatment can improve DT, but not longevity.

Materials and methods

Mature seeds of *S. virgata* were collected from 12 trees at Lavras (21˚22''S, 45˚1''W, Minas Gerais, Brazil) and stored in a cold room at 4˚C. Prior to germination tests, seeds were immersed in concentrated sulphuric acid (H_2SO_4) for 30 min and washed with abundant running water to remove physical dormancy. Germination assays were carried out in moist rolled paper, at 30˚C, under constant light (30 W m^{-2}) for ca. 36 h.

To determine the moment of loss of DT, three replicates of 20 seeds were selected according to their protruded radicle length (1 mm, 3 mm and 5 mm) and dried for 3 days. Throughout the study, drying treatments were performed by placing the seeds 3 days at 40% relative humidity (RH) at 22˚C, resulting in a water content as low as 0.14 g H_2O g^{-1} dry weight (Fig 5.1). Water content was assessed gravimetrically for triplicate samples of 10 seeds, by determination of the fresh weight and subsequent dry weight, after 18 h in an oven at 105˚C. Water content is expressed on a dry weight basis. After drying, seeds were

pre-humidified in air of 100% RH for 24 h at 22˚C in the dark to prevent possible imbibitional damage and subsequently rehydrated in water on a Copenhagen Table under a 12/12 h dark/light regime at 22˚C. Germinated seeds were evaluated according to the survival of their primary root, presence of green cotyledons (cotyledon survival) and growth resumption with both green cotyledons and development of a (secondary) root system (seedling survival).

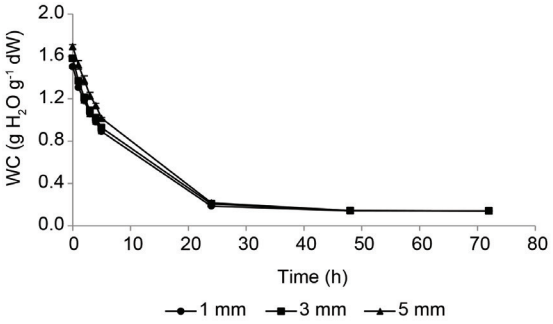


Fig 5.1 *Changes in water content upon dehydration of germinated* S. virgata *seeds at 40% RH with 1 mm, 3 mm, and 5 mm protruded radicles. Each data point is the average of three replicates of 20 seeds.* Bars *represent standard error.*

The re-establishment of DT in sensitive seeds was evaluated in three replicates of 20 germinated seeds selected according to their protruded radicle length (1 mm and 3 mm). These germinated seeds were dried (control) or treated with an osmoticum, namely incubation in 20 ml PEG solution with an osmotic potential ranging from -1.5 to -3.0 MPa at 4˚C or 22˚C (Villela and Beckert, 2001) in the dark, for 72 h. Possible hypoxic conditions were avoided by using an amount of PEG solution enough to cover the radicles, but not entire seeds. After incubation in PEG, seeds were rinsed thoroughly in distilled water, dried, pre-humidified, rehydrated and evaluated as described above.

Longevity was evaluated in triplicates of 15 germinated seeds with 1 mm protruded radicle and dried as described above. These seeds were stored in sealed plastic bags for 3 to 8 months at 4˚C. Additionally, longevity was estimated based on survival after an accelerated aging assay consisting of storage for two to seven days at 80% RH and 40˚C in the dark. Survival was evaluated as described above and by a tetrazolium

test. For the tetrazolium test, three replicates of six seeds were moistened on filter paper in Petri dishes for 24 h at 22˚C. After the removal of the seed coat, they were soaked in 0.5% (w/v) 2,3,5-triphenyltetrazolium chloride solution for 2 h in the dark at 30˚C and scored using location of staining as criteria (Camargos et al., 2008).

In order to evaluate the influence of a treatment to re-establish DT on longevity, germinated seeds with 1 mm protruded radicle incubated in -2.5 MPa PEG at 4˚C and dried were subjected to the same longevity tests as described for non-treated seeds.

Data were statistically analysed with SPSS 22.0 for Windows (IBM Corporation, Somer, NY, USA) using one-way ANOVA followed by Duncan post-hoc test ($P \leq 0.05$).

Results

Seeds of more than 90% of angiosperm species for which data are available are orthodox (Royal Botanic Gardens Kew, 2008). During germination of these seeds, water is taken up and metabolic processes are resumed, leading to the progressive loss of DT (reviewed by Dekkers et al., 2015). The point during germination when DT is lost varies between species. For example, within legume species, *Copaifera langsdorfii* and *Pisum sativum* lose DT before radicle protrusion (Pereira et al., 2014; Reisdorph and Koster, 1999), while *Glycine max*, *Medicago truncatula* and *Peltophorum dubium* lose DT after radicle protrusion (Buitink et al., 2003; Guimarães et al., 2011; Senaratna and McKersie, 1983). Here we show that *S. virgata* (also a legume) loses DT progressively after radicle protrusion (Fig 5.2) and, when the protruded radicle length reaches 5 mm, DT is almost completely lost.

Studies with model species have shown that after DT is lost, it can be re-established in a small developmental window by treatment with an osmoticum and/or ABA (reviewed by Dekkers et al., 2015). Fig 5.3 shows the percentage of DT obtained at different water potential/temperature combinations for germinated seeds with 1 mm and 3 mm protruded radicle. The water potential had considerably more impact on the re-establishment of DT than the temperature. For germinated seeds with

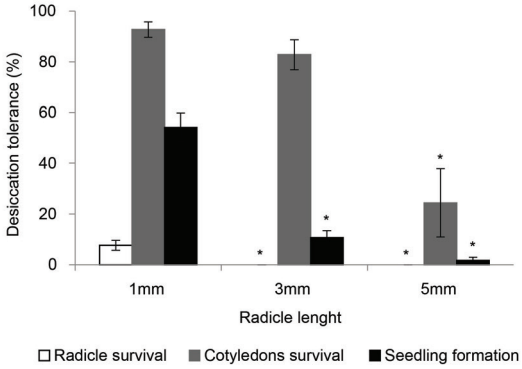


Fig 5.2 *Desiccation tolerance in germinated* S. virgata *seeds with different protruded radicle lengths. Each data point if the average of three replicates of 20 seeds.* Vertical bars *represent standard errors.* Asterisks *indicate significant differences* (P ≤ *0.05) comparing to germinated seeds with 1 mm protruded radicle.*

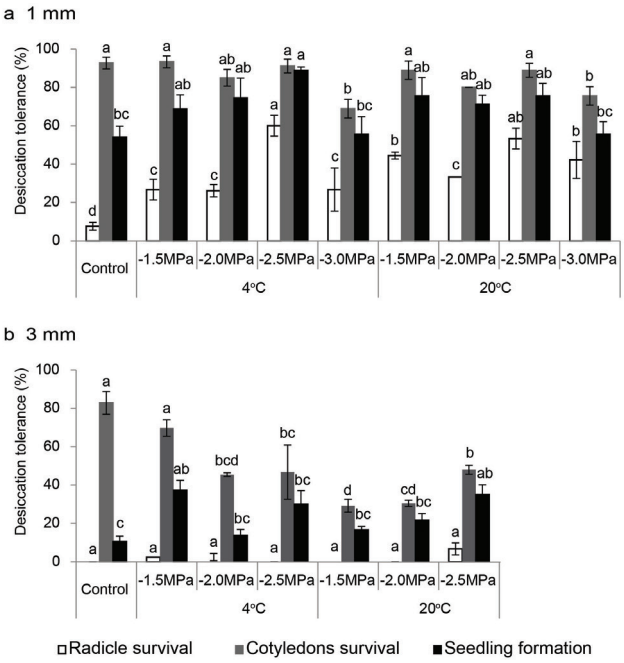


Fig 5.3 *Osmotic potential and temperature effects on the re-establishment of desiccation tolerance of germinated* S. virgata *seeds.* a *Germinated seeds with 1 mm protruded radicle.* b *Germinated seeds with 3 mm protruded radicle. Each data point is the average of three replicates of 20 seeds.* Bars *represent standard errors.* Control *represents germinated seeds dried directly at 40% RH without previous osmotic treatment.* Different letters *above bars indicate significant differences* (P ≤ *0.05).*

1 mm protruded radicle length, a water potential of -2.5 MPa led to the highest percentage of radicle survival. Still, even under these conditions, DT could not be re-established in all radicles. At the lowest water potential tested (-3.0 MPa), DT was observed in the lowest percentage of seeds. For germinated seeds with 3 mm protruded radicle length, some of the osmotic treatments significantly improved DT compared to non-treated seeds with the same radicle length, but the maximal percentage of seedling formation was lower than 40% and radicle survival was not improved. Re-induction of DT by incubation in ABA was unsuccessful, as ABA was not effective in arresting growth (data not shown). Based on these results, the best radicle length and treatment to re-establish DT in germinated *S. virgata* seeds is 1 mm radicle length and incubation in an osmoticum of -2.5 MPa at 4˚C.

Seed longevity can be analysed by storage and artificial aging. One of the methods to test artificial aging is the accelerated aging test, during which seeds are incubated at high humidity and temperature (Walters et al., 2005). We used cold storage (storage at 4˚C) and an accelerated aging test to determine longevity of germinated seeds with 1 mm protruded radicle and of these seeds incubated in an osmoticum at -2.5 MPa at 4˚C (Fig 5.4). The radicle was the most sensitive organ with respect to aging and the osmotic treatment did not significantly improve its survival. The cotyledons were the most tolerant organs. The osmotic treatment significantly improved cotyledon survival and seedling formation after accelerated aging for 4 days, but this positive effect was not observed in any of the other treatments. Overall, the osmotic treatment had a positive effect on seedling formation before aging, but was not able to improve longevity of the seeds.

The tetrazolium test (Suppl Fig 5.1) corroborates these results. In the treatments with high seedling survival, the staining was homogeneous from the radicle tip to the cotyledons in most of the seeds. Low seedling survival was correlated with a bigger extent of non-stained areas starting from the radicle tip and reaching the hypocotyl.

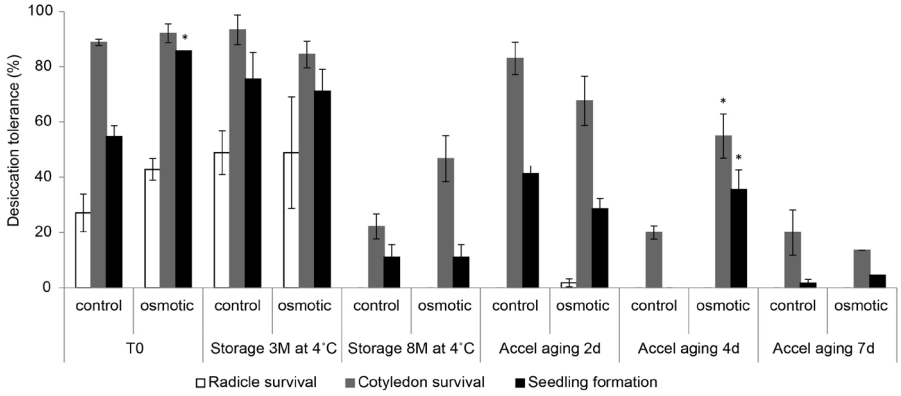


Fig 5.4 *Desiccation tolerance after storage or accelerated aging test (*Accel aging*) of germinated* S. virgata *seeds with 1 mm protruded radicle. Storage was performed at 4˚C for 3 and 8 months (*M*). Accelerated aging was performed for 2, 4 and 7 days (*d*). Each data point is the average of three replicates of 15 seeds.* Bars *represent standard error.* T0 *indicates germinated seeds dried directly at 40% RH without previous osmotic treatment.* Asterisks *indicate significant differences at* $P \leq 0.05$ *comparing control and seeds treated with an osmoticum (-2.5 MPa at 4˚C) within the same aging treatment.*

Discussion

Seed DT and longevity are crucial for long-term survival of orthodox seeds after dispersal (Waterworth et al., 2015). As a result, both longevity and DT share several mechanisms. For example, mechanisms responsible for protection of cellular macromolecules and cellular structures in the dry state, damage repair during germination and the minimization of oxidative stress damage are well documented in relation to both DT and longevity (Rajjou and Debeaujon, 2008; Waterworth et al., 2015).

More than 50% of germinated *S. virgata* seeds with 1 mm protruded radicle are able to survive desiccation without any previous treatment. As the radicle grows to 3 mm, there is a considerable drop in seedling formation after de- and rehydration to around 10%. Germinated seeds with 5 mm protruded radicle are sensitive to desiccation. DT is first lost by the radicle, the most sensitive organ in seeds of *S. virgata* and in other species (Buitink et al., 2003; Maia et al., 2011). The cotyledons are the most resistant organs, but the crucial point to determine if the germinated seed will survive is the ability to produce lateral roots

(Bruggink and van der Toorn, 1995; Maia et al., 2011). Germinated seeds are able to produce lateral roots when there are viable tissues that can differentiate to root primordia, as shown by the tetrazolium staining. When the non-stained areas included the hypocotyl, the germinated seeds did not produce root primordia and survival of the whole seed was compromised. On the other hand, when the hypocotyl was stained, lateral roots were produced to replace the main root and to enable the seedling to establish.

It has been shown that DT can be re-induced in germinated seeds by incubation in PEG and/or ABA (Bruggink and van der Toorn, 1995; Buitink et al., 2003; Maia et al., 2014). Attempts to re-induce DT in germinated *S. virgata* seeds by incubation in ABA failed, as ABA was not effective in arresting growth (data not shown). Re-induction of DT was possible by an osmotic treatment in germinated seeds with 1 mm protruded radicle. In these seeds, the combination of osmotic treatment with low temperature was optimal, but not critical. The osmotic treatment might inhibit radicle growth until enough ABA has accumulated to effectively induce DT (Buitink et al., 2003).

The osmotic treatment improved DT in germinated *S. virgata* seeds with 3 mm protruded radicle, but DT could not be fully rescued. DT is a complex trait and it is possible that in *S. virgata*, as the radicle grows, the mechanisms needed for DT are progressively lost and cannot be fully re-activated anymore.

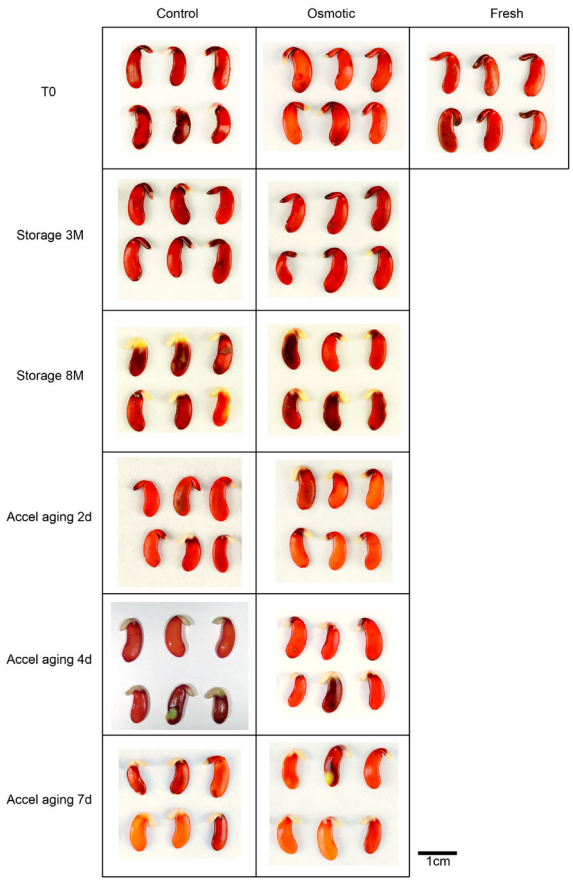
Germinated seeds could be stored for 3 months at 4˚C without significant losses in viability. Overall, longevity was not improved by the osmotic treatment. During seed development, the acquisition of DT precedes acquisition of longevity as a way to ensure timely activation of mechanisms needed for survival in the dry state (Verdier et al., 2013).

In conclusion, once DT is lost upon germination, an osmotic treatment can re-establish it up to a certain point during development. The same may not be true for longevity. More studies are necessary to unveil the molecular similarities and differences between DT and longevity.

Acknowledgments

The authors received financial support from the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Brazil).

Supplementary Data



Suppl Fig 5.1 Tetrazolium staining of germinated *S. virgata* seeds with 1 mm protruded radicle after storage or accelerated aging test (*Accel aging*). Storage was performed at 4˚C for 3 and 8 months (*M*). Accelerated aging was performed for 2, 4 and 7 days (*d*). Fresh seeds were imbibed, decoated and stained with tetrazolium. Two seeds per replicate are shown.

Chapter
Six

New insights into desiccation tolerance: analysis of the genome, transcriptome and proteome of the resurrection species *Xerophyta viscosa*

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In preparation

Abstract

Desiccation tolerance (DT) is the ability of an organism to withstand the loss of most of its water without dying. About 320 species of vascular plants, termed "resurrection plants", are desiccation tolerant. *Xerophyta viscosa* is one of the best studied resurrection species. *X. viscosa* seeds lose DT during germination and seedlings re-acquire it progressively during development. A treatment with the plant hormone abscisic acid (ABA) can induce DT in shoots, but not roots, of desiccation sensitive seedlings prematurely. Here, we used high throughput transcriptome (RNA-Seq) and proteome (iTRAQ) analysis to better understand the effect of the ABA treatment on shoots and roots of these seedlings. To improve transcript and protein identification, we also sequenced the genome of *X. viscosa*. *X. viscosa* is likely hexaploid with 48 chromosomes. The genome sequence comprises 260 Mb fragmented over 49,794 scaffolds with an N50 of 19 Kb. A normalized RNA library revealed ca. 41,500 transcripts from ca. 37,250 genes, of which ca. 24,000 showed homology to proeteins in the SwissProt database and ca. 10,500 were differentially expressed. A total of 1,900 proteins was identified via iTRAQ analysis. Differences in activation and repression of ABA signalling components, the cross talk between ABA and ethylene and senescence-related genes are presented as specific for shoot and root responses. A genome-wide identification of late embryogenesis abundant (LEA) proteins revealed 87 LEA encoding genes in the *X. viscosa* genome. Differences in the accumulation of LEA proteins are shown as a likely key for DT in shoots and roots.

Keywords

RNA-Seq; iTRAQ; polyploid; ABA; LEA

Introduction

Plants differ remarkably in their ability to tolerate water loss. The most tolerant plants are called "resurrection plants" and comprise 1,300 species of vascular plants (including epiphytic filmy ferns) (Gaff, 1971; Porembski and Barthlott, 2000). Resurrection flowering plants are even rarer and only about 135 species have been described so far (Black and Pritchard, 2002; Gaff and Oliver, 2013; Porembski and Barthlott, 2000). Resurrection plants survive almost complete dehydration (desiccation); they tolerate losing more than 90% of their relative water content (RWC) (Black and Pritchard, 2002; Porembski, 2011). Investigating the mechanisms of desiccation tolerance (DT) in resurrection plants is important to understand possible paths for the evolution of DT. In addition, they can be a source of genetic information to increase drought-tolerance in crops.

Although it is difficult to identify clear patterns in the systematic distribution of resurrection flowering plants, the family Velloziaceae comprises most desiccation tolerant species (Porembski, 2011). The Velloziaceae family is composed of 10 genera, of which 6 have species with desiccation-tolerant foliage (Franklin de Melo et al., 1997; Gaff and Oliver, 2013). Velloziaceae species can be found from sea level up to above 2,200 m, mainly in rocky outcrops of China, Saudi Arabia, Africa and South America (Mello-Silva et al., 2011; Porembski and Barthlott, 2000). Cytological analyses suggested that Velloziaceae species from South America (genus *Vellozia*, *Barbacenia* and *Barbaceniopsis*) are diploid (most of them) or tetraploid, while the African and Saudi Arabian species (genera *Xerophyta*) are mainly hexaploid (Franklin de Melo et al., 1997).

Xerophyta viscosa is a resurrection plant that has been used as a model to study DT (Farrant et al., 2015). *X. viscosa* is endemic to South Africa, Lesotho and Swaziland (Gaff, 1971; Farrant et al., 2015). The adult plants are able to withstand losing about 95% of their cellular water and resume metabolism within 72 h after water becomes available again (Farrant, 2000; Farrant et al., 2015). *X. viscosa* plants are poikilochlorophyllous (PDT) resurrection plants, as they activate a series of protection mechanisms, including the loss of most of their chlorophyll

and dismantling of the photosynthetic apparatus during dehydration (Farrant, 2000; Farrant et al., 2015). In this way, they develop all signs of advanced senescence but, upon rehydration, the photosynthetic machinery recovers and the assimilation of CO_2 resumes (Tuba and Lichtenthaler, 2011).

Despite the visual resemblance of the desiccation-induced breakdown of the photosynthetic apparatus in PDT plants and leaf senescence, PDT plants are able to avoid senescence during dehydration (Griffiths et al., 2014). It has been speculated that, in these plants, the UPR (unfolded protein response) and cell death pathways are modified. Also, the transcription factor ERD15 (EARLY RESPONSE TO DEHYDRATION 15) is attenuated and the endoplasmic reticulum stress signalling response modulated (Griffiths et al., 2014). Interestingly, older leaves and the tissue at the tip of the leaves die upon drying, suggesting that DT has an age-specific component and, indeed, genes associated with repression of drought-induced senescence are not expressed in older leaves (Beckett et al., 2012; Griffiths et al., 2014).

The phytohormone abscisic acid (ABA) is a stress-related signalling molecule well known to coordinate plant responses to numerous stress conditions leading to water deficit (Hauser et al., 2011). In resurrection plants, ABA activates a series of adaptive responses upon dehydration. For example, in rhizomes of *Polypodium vulgare*, an ABA treatment induced the synthesis of soluble sugars and changes in membrane permeability and ion accumulation, aiding osmotic adjustment (Bagniewska-Zadworna et al., 2007). Likewise, the relative expression of dehydrin genes was highly induced in leaves and roots of the resurrection species *Craterostigma plantagineum* upon ABA treatment, contributing to cellular protection (Giarola et al., 2015).

Another key aspect for DT is the synthesis and accumulation of late embryogenesis abundant (LEA) proteins. LEAs are intrinsically disordered proteins which have several possible protective functions in DT, such as hydration buffer, molecular shield, enzyme protectant, metal ion binding, antioxidant and membrane stabilizer (Tunnacliffe and Wise, 2007). During desiccation, transcripts of several LEAs were shown to accumulate in leaves of resurrection plants (Collett et al., 2004; Ndima et al., 2001). In *Xerophyta humilis*, 16 LEAs were identified in a cDNA

library of root and leaf tissues during the dehydration-rehydration cycle (Collett et al., 2004). However, a genome-wide analysis of LEA protein encoding genes in a resurrection species has so far not been done.

Here, we show that *X. viscosa* seeds lose DT upon germination, resulting in desiccation sensitivity of the newly formed seedlings. DT is reacquired only later during seedling growth, but can be induced earlier in shoots, but not in roots, by an ABA treatment. We assessed the changes in shoot and root transcriptomes and proteomes that occur during incubation of *X. viscosa* seedlings in ABA in relation to the resurrection phenotype. Moreover, to increase gene and protein identification, we combined paired-end short reads from Illumina and long PacBio reads from genomic DNA to generate a draft genome sequence of *X. viscosa*.

Materials and methods

Plant material and assessment of DT in seedlings

Seeds were harvested from *X. viscosa* plants collected in the Buffelskloof Nature Reserve (Mpumalanga Province, South Africa) and cultivated under glasshouse conditions at the University of Cape Town (South Africa). Seeds were sown on two layers of blue filter paper (Blue Blotter Paper, Anchor Paper Company) and 50 ml of distilled water and incubated in germination cabinets with constant white light at 25˚C for ~10 days.

To assess DT in *X. viscosa* germinated seeds or growing seedlings, they were selected by their developmental stage: (I) radicle protrusion, (II) emission of first leaf, and (III) first leaf between 1 and 2 mm length (Fig 6.1). Three replicates of 20 seedlings for each developmental stage were either fast-dried or slow dried in the presence of light. Fast drying was achieved by drying under an atmosphere of 32% relative humidity (RH) for 3 days at 20˚C, resulting in final water contents (WC) as low as 0.126 g H_2O g^{-1} dry weight (dw). Slow drying was achieved by drying under an atmosphere of 95% RH for 3 days followed by 3 more days under an atmosphere of 32% RH at 20˚C, resulting in a final WC down to 0.143 g H_2O g^{-1} dw (Suppl Fig 6.1). Water contents were assessed

gravimetrically by determination of the fresh weight and subsequently dry weight after 17 h at 105˚C. After dehydration, seedlings were pre-humidified in humid air (100% RH) for 24 h at 22˚C and rehydrated in H_2O at 22˚C on a Copenhagen Table under a 12/12 h dark/light regime. Seedlings that continued their development, emitted new leaves and developed a root system were considered desiccation tolerant.

Next, we tried to increase survival of fast drying by incubating the seedlings in solutions of ABA (5 µM, 10 µM and 50 µM), polyethylene glycol (PEG, -2.5 MPa and -3.5 MPa) and combinations of ABA and PEG before fast drying. To do so, seedlings in the developmental stage III were incubated for 3 days in 6-cm Petri dishes containing 1.3 ml of solutions of ABA, PEG and combinations of ABA and PEG on two sheets of white filter paper (grade 3 hw, Biolab Products, Sartorius Stedim Biotec) in the dark at 20˚C. After incubation, seedlings were rinsed in distilled water, transferred to new Petri dishes with one sheet of white filter paper, fast-dried and rehydrated as mentioned before. Seedlings were evaluated according to the survival of their primary root, ability to emit secondary root(s) and growth resumption with both green leaves and development of a root system.

Fig 6.1 Xerophyta viscosa *seedlings at different developmental stages. I – radicle protrusion, II – emission of first leaf, and III – first leaf between 1 and 2 mm length. Bar represents 1 mm.*

Cytogenetics

Chromosome preparations were done as described by Szinay et al. (2008). In summary, young leaves of *X. viscosa* were fixed in freshly prepared ice-cold Carnoy's fixative (acetic acid:ethanol, 1:3) at 4˚C until use. Fixed leaves were rinsed twice in distilled water and once in 10 mM sodium citrate buffer (pH 4.5). The leaves were then digested for

3h at 37˚C in an enzyme mix (1% cellulase RS (Yakult 203033, Yakult Pharmaceutical, Tokyo, Japan), 1% pectolyase Y23 (Sigma P-3026), and 1% cytohelicase (Bio Sepra 24970-014) in citrate buffer), carefully rinsed in sterile water, and placed on ice until use. A portion of the leaves was placed onto the middle of an alcohol-cleaned microscope slide with a few drops of water and chopped with fine needles. A drop of 60% acetic acid (30-40 µM) was added to the drop with the cell mixture and then the slide was placed on a 45˚C hot plate for 2 min. The drying drop was mixed using a needle every 15 s in order to spread the sample on the microscope slide. The slide was covered with 50 µl Carnoy's fixative, air-dried, post-fixed in 1% formaldehyde solution (in PSB, pH 6.8), air-dried again and stored at 4˚C until further use. The chromosome preparations were screened under a phase-contrast microscope and late-pachytene cells with little or no cytoplasm, good chromosome spreading and well-differentiated chromatin morphology were selected.

The assessment of DNA content of *X. viscosa* was performed by Iribov (Enkhuizen, The Netherlands). For genome size measurement, leaf tissue was mixed with 0.2 ml of nuclear extraction buffer solution (solution A of the plant high-resolution DNA kit, Partec GmbH, Munster, Germany) and incubated at room temperature for 15 min. The extracted nuclear solution was then stained with staining solution (10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP, 0.1% (v/v) Tritox X-100, Propidium Iodide) and analysed by flow cytometry on a CA-II cell analyzer (Partec). Tetraploid *Brassica oleraceae* was used as a reference for which the DNA content was calculated as 2.8 pg.

DNA extraction, genome assembly and annotation

X. viscosa plants used for DNA extraction were grown on soil in a greenhouse under a 8/16 h dark/light regime at 22°C day and 18°C night temperature. Genomic DNA was extracted from leaves from a pool of 5 plants as described by Bernatzky and Tanksley (1986) with modifications. Frozen leaves (2.5 g) were ground and mixed with 20 ml of extraction buffer (0.35 M Sorbitol, 100 mM Tris-HCl, 5 mM EDTA and 20 mM Na metabisulfite). The slurry was filtered through Miracloth (Calbiochem) and centrifuged for 15 min at 720 *g*. The supernatant was discarded

and the pellet was resuspended in 25 ml of extraction buffer with 0.4% Triton X-114 and centrifuged for 15 min at 720 *g*. The supernatant was discarded again and the pellet was once more resuspended in 25 ml of extraction buffer with 0.4% Triton X-114 and centrifuged for 15 min at 720 *g*. Then, the pellet was resuspended in 1.25 ml of extraction buffer, 1.75 ml of nuclei lysis buffer pH 7.5 (200 mM Tris-HCl, 50 mM EDTA, 2 M NaCl and 2% CTAB) and 0.6 ml of 5% Sarcosyl. Next, 5 µl RNase A (10 mg/ml) was added to the mixture, that was heated to 65˚C for 20 min. The lysate was mixed with 8 ml of chloroform/isoamylalcohol (24:1) and centrifuged for 15 min at 3,220 *g* without brakes. The aqueous phase was transferred to a new tube and mixed with the same amount of cold isopropanol. After 5 min of precipitation at room temperature, the solution was centrifuged for 30 min at 3,220 *g* at 4˚C. The pellet was washed with 2 ml of 75% ethanol, centrifuged for 5 min at 3,220 *g* at 4˚C. After drying, the pellet was resuspended in 20 µl of 10 mM Tris-HCl buffer pH 8.0.

A library (~300 bp insert size) was constructed and sequenced paired end with Illumina HiSeq (100 bp, ~77x coverage). In addition, long PacBio reads were produced for scaffolding (~5x coverage, 3.3 kb average read length). The Illumina and PacBio reads were assembled using dipSPAdes, an algorithm for assembling highly polymorphic genomes (Safonova et al., 2014), and additional scaffolding was done using SSPACE3-0 (SSAKE-based Scaffolding of Pre-Assembled Contigs after Extension) (Boetzer et al., 2011). PBJelly was used for gap filling and genome improvement using the PacBio long reads (English et al., 2012). The MAKER2 annotation pipeline (Holt and Yandell, 2011) was applied for gene prediction and repeat annotation. The *ab initio* gene prediction tools AUGUSTUS (Stanke and Morgenstern, 2005) and SNAP (Korf, 2004) were trained on the transcriptome data (see below). Predicted genes were functionally annotated by a consensus approach using InterProScan (Mitchell et al., 2014), Gene Ontology (The Gene Onthology Consortium, 2015), Kyoto Encyclopedia of Genes and Genomes (KEGG, Ogata et al., 1999), Swissprot and Translated EMBL Nucleotide Sequence Data Library (TrEMBL, Boeckmann et al., 2003), and Blast2GO (Conesa et al., 2005). SNPs (Single Nucleotide Polymorphisms) and INDELs (Insertions and Deletions) were called relative to the genome

using FreeBayes (version 0.9.18-25-g5781407, Garrison and Marth, 2012) with the following parameter settings: --ploidy 6, --min-base-quality 0, --min-mapping-quality 5, --min-alternate-fraction 0.1, --min-alternate-count 3. Variants with quality less than 20 were discarded. The raw data (Illumina and PacBio) was deposited in the short read archive (SRA) under SRS1041365.

The chloroplast genome was assembled separately using the IOGA (Iterative Organelle Genome Assembly) pipeline (Bakker et al., 2015) with the chloroplast genome of *Phalaenopsis equestris* as a reference (Jheng et al., 2012). All reads were mapped to the chloroplast genome using Bowtie2 (Langmead and Salzberg, 2012) and manual curation was performed when needed. CpGAVAS was used to annotate the genome (Liu et al., 2012). Manual inspection and curation were performed to resolve conflicting annotations. TopHat was used to align all transcriptome libraries to the chloroplast using default settings (Trapnell et al., 2012).

RNA extraction, sequencing and assembly

For RNA extraction, 3 replicates of 20 seedlings in stage III treated with ABA (as described before) and non-treated seedlings in the same developmental stage were dissected in three parts: seed coat, shoots and roots. The seed coat was discarded and shoots and roots were used for RNA extraction. Total RNA was extracted following a modified hot borate protocol (Wan and Wilkins, 1994). The seedling parts were ground and mixed with 800 µl of extraction buffer (0.2 N Na borate decahydrate (Borax), 30 mM EGTA, 1% SDS, 1% Na deoxycolate) containing 1.76 mg DTT and 52.8 mg PVP40, and heated to 80˚C. Then, 4 mg proteinase K was added to this solution before incubation for 15 min at 42˚C. After the addition of 64 µl of 2 M KCL, the samples were incubated on ice for 30 min and subsequently centrifuged for 20 min at 12,000 *g*. The supernatant was transferred to a new tube, 260 µl of ice-cold 8 M LiCl was added, and the tubes were incubated overnight on ice. After centrifugation at 4˚C for 20 min at 12,000 *g*, the pellets were washed with 750 µl of ice-cold 2 M LiCl and re-suspended in 100 µl milliQ water. The samples were phenol–chloroform extracted, DNAse treated (RQ1 DNase, Promega) and further purified with RNeasy spin columns (Qiagen) according to

the manufacturer's instructions. RNA quality and concentration were assessed by agarose gel electrophoresis and a NanoDrop ND-1000 spectrophotometer (Nanodrop® Technologies).

The samples were sequenced (150 nt, single-end reads) with Illumina HighSeq 2500 and 55.2 Gb were obtained after trimming (12 libraries). A *de novo* transcriptome assembly was constructed using Trinity (Grabherr et al., 2011). The raw RNA-seq data have been deposited in NCBI SRA database under accession SRS1071017.

The reads were mapped and quantified using TopHat and Cufflinks from the Tuxedo suite (Trapnell et al., 2012). Differential expression was then computed using Cuffdiff, also from the Tuxedo suite (Trapnell et al., 2012). The resulting gene set was used for an over-representation analysis (ORA) to recover over-represented biological processes (using Benjamini & Hochberg False Discovery Rate (FDR) correction, *q*-value ≤ 0.05) based on gene ontologies using the plugin BiNGO (Maere et al., 2005) for Cytoscape. InterProScan and protein BLAST (using *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays* as a reference) were used to assign GO-terms to the total set of annotated genes. The whole annotation was used as reference set. A GO term's semantic distance with respect to other semantically close terms ("Dispensability") was calculated using the online tool ReviGO (Supek et al., 2011) and used to remove redundant terms applying a cut-off of ≤ 0.05.

To verify the accuracy of the data, the expression levels of 12 genes was analysed by qPCR in shoots. Total RNA was extracted as described above for the RNA-Seq. The first strand cDNA was synthesized with 1 µg of total RNA using the iScriptTM cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Reverse transcription was performed at 37˚C for 40 min followed by 5 min at 85˚C. The cDNA was diluted 20 times and stored at -20˚C prior to further analysis by qPCR. The qPCR was performed in a total volume of 10 µl containing 2.5 µl of diluted cDNA, 0.5 µl of primers (10 µM), 5 µl of iQ SYBR Green Supermix (Bio-Rad) and 2 µl of milliQ water. The qPCR program run consisted of a first step at 95˚C for 3 min and afterwards 40 cycles alternating between 15 s at 95˚C and 1 min at 60˚C. Candidate reference genes were chosen based on the RNA-Seq data applying a cut-off of ≥ 0.9 for *q*-value. These genes were further checked using qBase+ (Biogazelle) applying a cut-

off of ≤ 0.5 for *M*-value (gene stability value) and ≤ 0.15 for *CV*-value (coefficient of variation), generating a shortlist of 7 reference genes. The 3 reference genes with most stable expression in the qPCR were used for expression normalization of the target genes. Both RNA-Seq and qPCR showed comparable trends (Suppl Table 6.1)

Protein extraction, labelling and annotation

For protein extraction, 4 replicates of 20 seedlings were prepared in the same way as for RNA extraction. Excised shoots and roots were used. Total proteins were phenol-extracted as described by Isaacson et al. (2006). Frozen material was ground in the presence of PVPP (10% wt/wt). The powder was suspended in 100 µl of cold extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl pH 7.5, 50 mM EDTA, 2% (vol/vol) β-mercaptoethanol and protease inhibitor (Roche)). An equal volume of phenol saturated with Tris-HCl pH 7.5 was added. The mixture was shaken for 30 min at 4˚C, centrifuged at 5,000 *g* for 30 min at 4˚C and the upper phenolic phase was transferred to a new microtube to which the same volume of extraction buffer was added (these steps were repeated). Again, the mixture was shaken for 30 min at 4˚C and centrifuged at 5,000 *g* for 30 min at 4˚C. The upper phenolic phase was transferred to a new microtube and 5 volumes of cold 0.1 M ammonium acetate in methanol was added. The mixture was stored at -20˚C overnight and centrifuged for 30 min at 5,000 *g* at 4˚C. The supernatant was carefully removed and discarded. The pellet was washed with 2 volumes (based on the volume of the last collected phenolic phase) of ice-cold methanol and centrifuged for 10 min at 5,000 *g* at 4˚C. The supernatant was carefully removed and discarded. These washing steps were performed two times using methanol and two more times using acetone. Finally, the pellet was dried in a fume hood under a gentle stream of nitrogen gas and resuspended in 60 µl of SDS buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% (wt/vol) SDS, 10% (vol/vol) glycerol). Protein sample concentration was assessed using the Micro BCA Protein Assay Kit (Pierce, USA).

Sample volumes were adjusted to 10 µl with 50 mM triethylammonium bicarbonate (TEAB) and reduced by the addition of

1µl 100 mM triscarboxyethyl phosphine (TCEP) in 50 mM TEAB followed by incubation for 1 h at 60˚C. After reduction, the samples were cooled to room temperature, cysteine residues were alkylated using 1 µl 5 mM methyl-methanethiosulfonate (MMTS, Sigma) and samples were incubated at room temperature for 30 min. Then, the samples were diluted with 50 mM TEAB to 45 µl and 5 µl trypsin (Promega) solution (1 mg/ml) was added. The samples were incubated overnight at 37˚C and reduced to 10 µl by Speed Vac. To the concentrated samples, 10 µl of 600 mM TEAB were added to a final concentration of 300 mM and the pH was confirmed as being greater than pH 7.5. The 8-plex iTRAQ (isobaric tags for relative and absolute quantification) reagents (AB Sciex) were prepared according to manufacturer instructions and distributed in vials. The vials were centrifuged and their content were added to the respective samples. After the samples were vortexed and centrifuged, all the tubes were incubated at room temperature for 2 h. To stop the reaction, 100 µl of MilliQ water was added to the samples and they were incubated at room temperature for 30 min. The volume of each sample was reduced to 50 µl by Speed Vac. Due to low protein concentration, pairs of sample replicates were combined, resulting in two biological replicates per treatment, and the final volume was reduced to 100 µl by Speed Vac.

For isoelectric point-based peptide separation, 3100 OFFGEL Fractionator and OFFGEL Kit pH 3-10 (both Agilent Technologies) with a 12-well setup were used according to the supplier's protocol. Ten min prior to sample loading, 13-cm-long immobilizer pH gradient (IPG) strips (Bio-Rad, USA) with a linear pH gradient ranging from 3 to 10 were rehydrated with 40 µl of focusing buffer per well. An estimated 120 µg of desalted iTRAQ labelled pooled peptide was diluted in focusing buffer to a final volume of 1.8 ml and 150 µl of sample was loaded in each well. Peptide focusing was performed up to a total of 20 kVh with a maximum voltage of 8,000 V and maximum current of 50 µA.

LC–MS/MS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system. The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (90% ACN, and 0.1% formic acid in water). The off gel fractionated peptides

were dissolved in sample loading buffer (5% Acetonitrile and 0.05% TFA in water) and loaded on a C18 trap column. Chromatographic separation was performed with an Acclaim Pepmap (Thermo Fisher Scientific, USA) C18 column (75 µm × 250 mm × 3 µm). The gradient was delivered at 250 nl/min and consisted of a linear gradient of mobile phase B initiating from solvent B: 8–25% over 70 min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 250°C. The applied electrospray voltage was 1.6 kV.

MaxQuant was used to analyse and retrieve the normalized intensity values for each replicate per peptide (Cox and Mann, 2008). The annotated *X. viscosa* proteins were used to assign peptides to proteins. Perseus (http://141.61.102.17/perseus_doku/doku.php?id=start, MaxQuant, v1.11, Martinsried, Germany) was used to filter the data and determine the reliable peptides.

Orthology between 12 other plant species (A*mborella tricopoda, A. thaliana, Elaeis guineensis, Eragrostis tef, Hordeum vulgare, Musa acuminate, O. sativa, Phalaenopsis equestris, Phoenix dactylifera, Physcomitrella patens, Spirodela polyrhiza, and Z. mays*) was determined with the OrthoMCL pipeline (Chen et al., 2006). The orthogroups generated were clustered into larger gene families (i.e. super-orthogroups, SOGs) by using the minimum E-value between all pairs of orthologroups as the input matrix for another MCL clustering using an inflation value of 3.5, as described in Amborella Genome Project (2013).

Analysis of ABA signalling, LEA protein and leaf senescence related genes

Protein sequences of genes related to leaf senescence (Li et al., 2012), ABA signalling network (Hauser et al., 2011) and LEA proteins (Hunault and Jaspard, 2010; Jaspard et al., 2012) were downloaded from TAIR10 (https://www.arabidopsis.org/, Lamesch et al., 2010) and UniProt (http://www.uniprot.org/, Wu et al., 2006). The sequences were compared with the full protein set of *X. viscosa* using BLASTP (Altschup et al., 1990). We considered best hits the ones with lowest E-value, with a threshold of $\leq 10^{-15}$, and those with a difference between their E-value and the lowest hit's E-value smaller than 10^{-10}. Then we compared the

sequences of these best hits of *X. viscosa* with sequences of plant genes (taxid 3193) available on the NCBI website using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The best hits (according to the same criteria already mentioned) in this second comparison were evaluated in relation to the original lists to determine the *X. viscosa* most likely orthologs of those genes.

The ABA signalling network was reconstructed using PathVisio3 (Kutmon et al., 2015) and analysed using the plug-in CyLineUp (http://apps.cytoscape.org/apps/cylineup) for Cytoscape.

Results

Desiccation tolerance in *X. viscosa* seedlings

Seeds of *X. viscosa* are desiccation tolerant, but just like seeds of other orthodox-seeded species, they lose DT during germination (Dekkers et al., 2015). DT is re-established gradually during subsequent development, presumably first in shoots and (much) later in roots. Tolerance to slow drying is gained earlier than tolerance to fast drying (Fig 6.2). Survival to fast drying was improved by treatment with PEG and ABA separately, but the combination of both did not appreciably improve survival (Suppl Table 6.2). The treatments with 5 µM and 50 µM ABA improved survival to fast drying of primary roots, yet, most of them remained sensitive. Treatment with 50 µM ABA was more effective in older seedlings (Fig 6.2). The ABA treatment did not improve tolerance to slow drying (data not shown). Therefore, we chose 50 µM ABA followed by fast drying for transcriptome and proteome analysis.

Genome sequencing, assembly and annotation

The genome sequence of *X. viscosa* comprises 260 Mb fragmented over 49,794 scaffolds with an alignment rate of 94.8% (Illumina PE), N50 of 19.0 kb, N90 of 3.0 kb and GC-content of 36% (Table 6.1a). *X. viscosa* has 48 small chromosomes (Suppl Fig 6.2) with an estimated length of 1.5-2.0 microns. It is likely to be hexaploid, but octoploidy

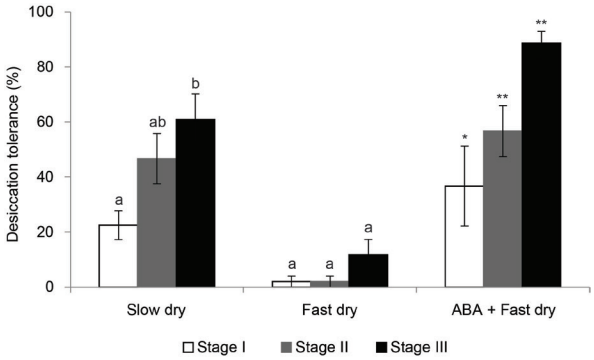

Fig 6.2 *Tolerance to slow and fast drying in seedlings of* X. viscosa *at three developmental stages. Fast drying was preceded or not by an ABA treatment. Slow drying was performed at 95% RH for 3 days followed by 3 more days at 32% RH. Fast drying was performed at 32% RH for 3 days. Treatment with 50 µM ABA lasted 3 days. Data points are averages of 5 replicates of at least 15 seedlings.* Bars *represent standard error. Different* letters *indicate significant differences (*P ≤ *0.05) after one-way ANOVA with Tukey post-hoc test comparing developmental stages in slow drying and fast drying treatments.* Asterisks *indicate statistical significant differences after Student's t test comparing fast drying with and without a previous ABA treatment at* P ≤ *0.05 for single and* P ≤ *0.01 for double asterisks.*

Table 6.1 *Overview of assembly, annotation and polymorphisms of the* X. viscosa *genome.*

a) Assembly	**Number**	**N50 (kb)**	**N90 (kb)**	**Alignment rate**
Contigs	55,592	16.31	2.35	-
Scaffolds	49,794	19.06	3.0	94.8%
b) Annotation	**Number**	**Mean length (bp)**	**Density**	**Percentage of the genome**
Protein coding genes	24,829	3,896	-	38%
Exons	134,401	273.11	5.41 exons/gene	14%
Introns	101,108	615.6	4.07 introns/gene	24%
rRNA	166	422.09	-	0.03%
snRNA	90	85.04	-	0.003%
tRNA	238	72.19	-	0.007%
Annotated repeats	561,916	-	-	34%
c) Polymorphisms	**Number**	**Density**		
SNPs	2,535,358	9.75 kb^{-1}		
INDELs	510,676	1.96 kb^{-1}		
Multi-allelic sites	209,326	0.8 kb^{-1}		

cannot be rejected because the percentage (supporting reads) of non-reference alleles shows a large peak around 12.5% (Suppl Fig 6.3). Using the CEGMA (Core Eukaryotic Genes Mapping Approach) pipeline, 79% and 94% of 248 core eukaryotic genes could be matched to the genome assembly completely and partially, respectively (Parra et al., 2007). Although the fragmentation of the assembly is substantial, these CEGMA scores indicate that the gene space is largely covered.

A total of 24,829 protein-coding genes supported by mRNA evidence were predicted (Table 6.1b). The largest number of genes exhibited homology with proteins in the TrEMBL (95%) and SwissProt (82.5%) databases. In addition, conserved protein domains could be identified in 80% of the predicted proteins using InterProScan. We annotated 34% (88.75 Mb) of the genome as repetitive (Table 6.1b) (Jurka et al., 2005). The largest group (14.6 Mb) consisted of long-terminal-repeat retrotransposons (LTR-Rs), mainly Gypsy-like and Copia-like. Non-LTR retrotransposons of the long interspersed nuclear element (LINE) type and short interspersed nuclear elements (SINEs) were represented by 0.51 Mb and 2.2 Mb, respectively.

Estimated average gene length was 3,986 bp, with on average 5.4 coding exons and 4.07 introns per gene (Table 6.1b). Homology-based annotation of non-coding RNA resulted in 166 predicted rRNAs, 90 snRNAs and 238 tRNAs. SNP calling identified 2,535,358 SNPs with a SNP density of 9.75 per kb^{-1} (Table 6.1c). We also identified 510,676 INDELs and 209,326 multi-allelic sites (sites with more than two alleles).

Based on the translated *X. viscosa* gene set and the protein sets of 12 other plant species, we inferred orthologous gene relationships (Table 6.2).

The chloroplast genome of *X. viscosa* comprises 155.5 kb and contains two pseudogenes and 90 predicted genes that can be further divided into 8 rRNAs and 31 tRNAs (Fig 6.3). Chloroplast genomes are in general between 120-160 kb long and contain around 130 genes (Liu et al., 2012).

Table 6.2 *Orthology assessment of thirteen plant species. In total, 4,692 super orthologous groups (SOGs) were created with 4,385 multi-species groups.*

Species	Family	# Proteins	# SOGs with *X. viscosa*	# Proteins in *X. viscosa* groups	# Single species SOGs
X. viscosa	Velloziaceae	24,829	-	-	2
P. patens	Funariaceae (Bryophyta)	32,271	3,352	18,714	71
A. tricopoda	Amborellaceae	27,313	3,715	17,725	59
E. guineensis	Arecaceae	39,450	3,802	36,449	2
E. tef	Poaceae	41,136	3,518	29,899	11
H. vulgare	Poaceae	26,319	3,707	21,042	6
M. acuminata	Musaceae	36,549	3,751	25,141	2
O. sativa	Poaceae	35,426	3,782	22,014	53
P. equestris	Orchidaceae	42,294	3,740	31,093	14
P. dactylifera	Arecaceae	28,889	3,628	20,891	1
S. polyrhiza	Araceae	18,888	3,190	13,107	6
Z. mays	Poaceae	36,436	3,526	26,062	62
A. thaliana	Brassicaceae	27,383	3,725	21,738	18

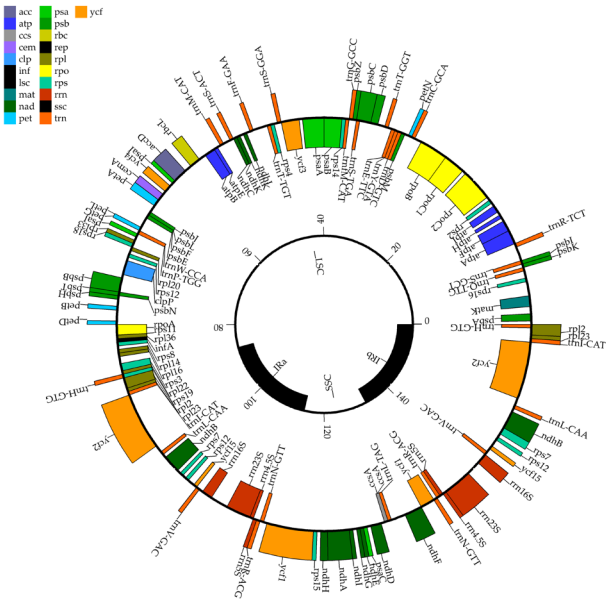

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Fig 6.3 *A circular map of the chloroplast genome of* X. viscosa.

Transcriptome and proteome

An RNA-Seq and an iTRAQ library were prepared from roots and shoots of *X. viscosa* seedlings in stage III before and after incubation in 50 μM ABA. The RNA-Seq library was used to generate a total of 55.2 Gb and revealed ca. 41,500 transcripts from ca. 37,250 genes. The overall alignment rate of the RNA-Seq reads with the genome was 81%. A total of 1,900 proteins were identified via iTRAQ analysis in the same samples. The sources of variation measured in the samples were estimated with PCA (Principal Component Analysis, Fig 6.4). A Pearson correlation analysis was used to calculate the association between the transcriptome and the proteome for each treatment. The correlation values varied from 0.40 (P-value ≤ 0. 5) to 0.45 (P-value ≤ 0.45). In total, 147 genes accumulated transcripts and proteins in shoots upon the ABA treatment. The GO terms enriched in these genes are mainly related to primary metabolism (*generation of precursor metabolites and energy* and *gluconeogenesis*) and reproduction (*reproduction, floral organ abscission* and *reproductive process*).

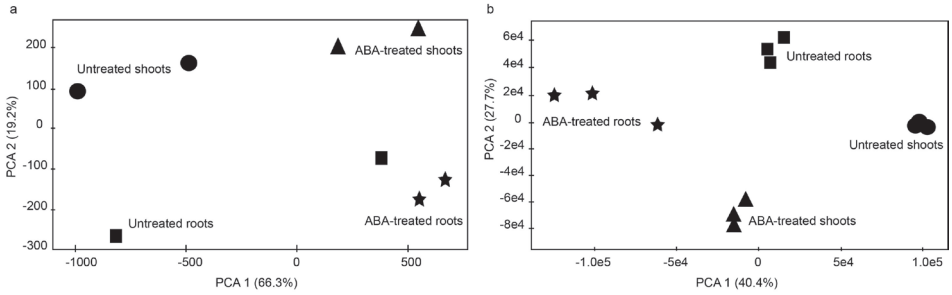


Fig 6.4 *PCA plots of* X. viscosa a *transcriptome and* b *proteome. The percentage of the variation explained by each component is specified between parentheses.*

A differential expression analysis generated a set of 14,780 gene transcripts differentially expressed comparing ABA-treated and untreated samples (q-value ≤ 0.05). These gene transcripts were divided in eight patterns according to their expression in shoots and roots (Fig 6.5a). A summary of the GO categories over-represented in each pattern can be found in Suppl Table 6.3. The GO category *ethylene biosynthetic process* was over-represented in the patterns "S↑,R↑" (S: shoots, R:

roots, ↑: higher expression) and "S→,R↑" (→: unchanged expression). GO categories related to growth and metabolism (e.g. *biosynthetic process*, *cell proliferation*, *cell wall organization or biogenesis*, *developmental maturation*, *growth*, *metabolic process*, *photosynthesis*, *primary metabolic process*, *regulation of growth* and *shoot system morphogenesis*) were over-represented in the patterns "S↓,R↓" (↓: reduced expression) and "S↓,R→". The GO category *regulation of oxygen and reactive oxygen species* was over-represented in the pattern "S↑,R↓". Genes in this category are in the same SOG as WRKY DNA-binding proteins (SOG14 and SOG2723), MAP kinases (SOG42) and calmodulins (SOG1902). The category *water transport* was over-represented in the pattern "S↓,R↓". Genes related to aquaporins, cellulose synthesis and glutamine synthesis are in the same SOGs (SOG309, SOG315, SOG518 and SOG2880) as genes in this GO category.

Most of the proteins (77%) identified by iTRAQ did not display significant changes in abundance ($P \leq 0.05$). Among the differentially expressed proteins, 90% accumulated in shoots (Fig 6.5b). The GO categories enriched in these proteins are mainly related to metabolism (e.g. *generation of precursor metabolites and energy*, *metabolic process*, *primary metabolic process* and *small molecule catabolic process*) and signalling (e.g. *flavonoid metabolic process*, *hydrogen peroxide metabolic process* and *oxygen and reactive oxygen species metabolic process*).

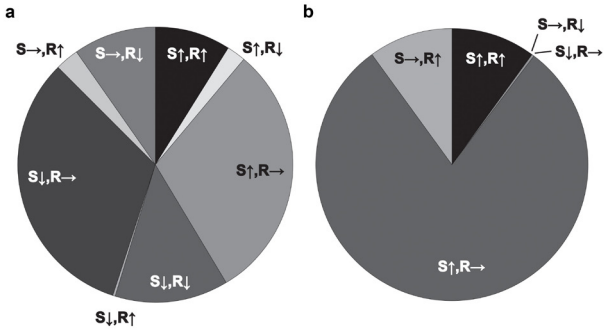


Fig 6.5 *Proportion of differentially expressed* a *transcripts and* b *proteins with a specific expression pattern, comparing ABA treated and untreated samples. S: shoots, R: roots, ↓: reduced expression, ↑: higher expression, →: unchanged expression.*

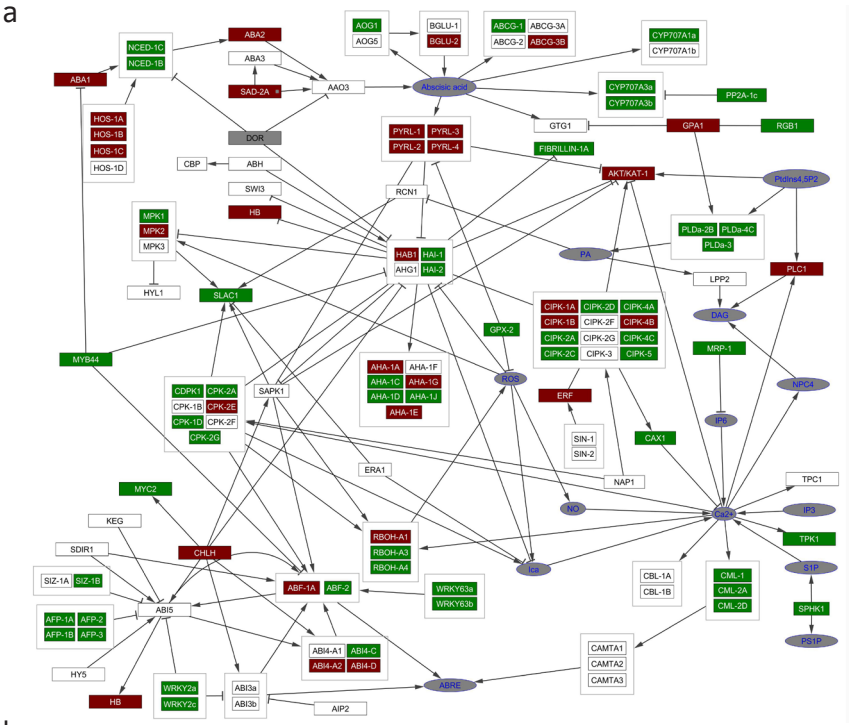
ABA signalling network

The ABA signalling components show remarkable land-plant specific conservation (Hauser et al., 2011; Komatsu et al., 2013; Sakata et al., 2009). Therefore, we searched for putative homologs of these genes in the genome of *X. viscosa* (Suppl Table 6.4). Moreover, we reconstructed the ABA signalling network in *X. viscosa* based on sequence similarity and plotted the transcriptional changes identified in shoots and roots in response to incubation in ABA (Fig 6.6). In general, a higher percentage of genes changed transcript abundance in shoots than in roots. Genes related to ABA biosynthesis displayed declined transcript abundance in shoots, while genes related to ABA catabolism increased their expression in shoots and roots. The transcription factor MYB44 showed increased transcript abundance in shoots whereas the ABA receptors PYRL (PYRABACTIN RESISTANCE (PYR)-LIKE) showed decreased transcript abundance in shoots, but not in roots. Transcript abundance of negative regulators of ABI5 (ABA INSENSITIVE 5) declined in shoots. WRKY genes and the Group A PP2C phosphatases HAI-1 and HAI-2 showed transcript accumulation in shoots, but not in roots. A higher number of genes which are positively regulated by Ca^{2+}, displayed higher transcript abundance in shoots than in roots.

LEA proteins

LEA protein genes have been largely correlated with responses to desiccation, salt and cold stress in a variety of organisms, such as plants, invertebrates and microorganisms (Tunnacliffe and Wise, 2007). Computational analysis of the amino acid sequences of LEA proteins to get novel insights into molecular and functional characterization of LEA

Fig 6.6 *ABA signalling network (based on Hauser et al., 2011) reconstructed for* X. viscosa ► *based on sequence similarity.* a *Transcript changes in shoots;* b *Transcript changes in roots.* Red rectangles *indicate genes significantly declining transcript abundance after ABA treatment.* Green rectangles *indicate genes significantly accumulating transcripts.* White rectangles *indicate genes without significant changes in transcript abundance.* Grey rectangles *indicate components without a homolog in* X. viscosa. Grey ellipses *indicate components that are not genes.* ← *indicate positive regulation.* ┣ *indicate negative regulation.*



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genes were facilitated by the release of LEAPdb (Hunault and Jaspard, 2010). We took advantage of this database to search for *X. viscosa* genes with high similarity with LEA genes. We identified 87 putative LEA protein genes in the genome of *X. viscosa* (Suppl Table 6.5), of which 58 have the highest homology with LEA proteins from *Eucalyptus grandis*, 38 with *A. thaliana* and 36 with *Glycine tomentella* LEAs. 62 of the putative *X. viscosa* LEA protein genes were differentially expressed in the transcriptome dataset and 19 in the proteome dataset (Fig 6.7).

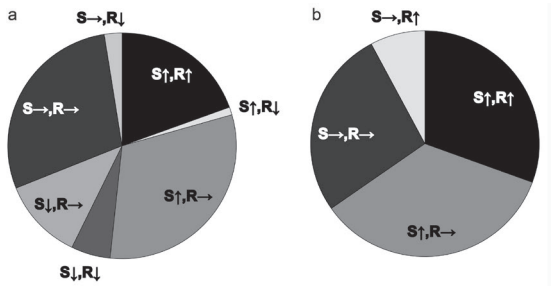

Fig 6.7 *Proportion of putative LEA protein genes with a specific differentially expression pattern comparing ABA treated and untreated samples.* a *Transcriptional changes;* b *Protein changes. S: shoots, R: roots, ↓: reduced expression, ↑: higher expression, →: unchanged expression.*

Senescence-related genes

In many non-resurrection plants, the activation of osmotic mechanisms to enable water retention is an essential part of surviving water-deficit (Griffiths et al., 2014). When water loss becomes more severe, they initiate leaf senescence. Resurrection plants, on the other hand, initiate similar osmotic responses during dehydration, but avoid senescence (Griffiths et al., 2014). Therefore, we investigated the presence and expression patterns of genes known to promote or delay senescence in our datasets (Li et al., 2012). We found 193 putative homologs in the *X. viscosa* genome (97 differentially expressed in our transcriptome dataset) for 62 of the 74 *A. thaliana* genes known to promote leaf senescence (Table 6.3, Suppl Table 6.6). We also found 138 putative homologs (52 differentially expressed in our transcriptome dataset) for 59 of the 85 *A. thaliana* genes known to delay leaf senescence. The GO categories enriched in *X. viscosa* genes that putatively promote

senescence and which show declined transcript abundance in shoots are mainly related to cell homeostasis (*chemical homeostasis* and *di-, tri-valent inorganic cation homeostasis*) and cell division (*mitotic interphase* and *G1/S transition of mitotic cell cycle*). The GO categories enriched in *X. viscosa* genes that putatively delay senescence and accumulate transcripts in shoots are mainly related to senescence (*senescence* and *leaf senescence*), transcription regulation (*signalling* and *signal transduction*) and amino acid modification (*peptidyl-histidine phosphorylation* and *branched-chain amino acid catabolic process*).

Table 6.3 *Number of* X. viscosa *homologs of genes related to senescence in* A. thaliana *(Li et al., 2012) differentially expressed in the* X. viscosa *transcriptome dataset.*

		X. viscosa homologs
Promote senescence in *A. thaliana*	Accumulate transcripts in shoots	58
	Accumulate transcripts in roots	28
	Decline transcripts in shoots	29
	Decline transcripts in roots	16
Delay senescence in *A. thaliana*	Accumulate transcripts in shoots	21
	Accumulate transcripts in roots	7
	Decline transcripts in shoots	25
	Decline transcripts in roots	8

Discussion

Harsh environmental conditions have led to the development of distinctive plant traits that attract considerable scientific interest (Porembski and Barthlott, 2000). Vegetative DT, for example, might have evolved in response to the rigors of being dry in marginal habitats (Oliver et al., 2000). In such environments, being polyploid could aid survival by potentially allowing for ecological niche expansion or increased flexibility in the organism's responsiveness to an unstable environment (Madlung, 2013).

At least 10 of the 45 species in the genus *Xerophyta* are desiccation tolerant and all the species studied so far are hexaploid (Farrant et al., 2015; Franklin de Melo et al., 1997; Mello-Silva et al., 2011). *X. viscosa* is largely found hanging off cliff edges in rocky terrain or inselbergs in exposed grasslands frequently subjected to periods of

severe water deficit (Farrant et al., 2015). In this environment, being desiccation tolerant and polyploid may thus imply ecological advantage.

Mature seeds and adult plants of *X. viscosa* are desiccation tolerant. Yet, newly germinated seeds are desiccation sensitive, constituting a very interesting system to study DT. *X. viscosa* seedlings first acquire tolerance to slow drying and later to fast drying (Fig 6.2). It is possible that during fast drying there is insufficient time to fully activate protection mechanisms that are constitutively present in adult leaves, resulting in lethal damage (Farrant et al., 1999). Additionally, adult leaves have morphological mechanisms that might slow-down drying that are not present in young seedlings. An ABA treatment may induce tolerance to fast drying early in leaves of seedlings (Fig 6.2), suggesting a priming effect to respond to desiccation. However, most of the roots remain sensitive. Considering that ABA is a well-known mediator of plant stress responses, our system represents a unique tool to deepen our understanding of the ABA-related mechanisms controlling DT. Therefore, we used high throughput transcriptome and proteome analysis to study the molecular changes induced by an ABA treatment in seedlings of *X. viscosa*. To achieve a high-quality annotation of transcripts and proteins, we also sequenced the genome of *X. viscosa*.

X. viscosa has 48 small chromosomes (Suppl Fig 6.2). Other related species (from the Velloziaceae family) were also reported to have small (i.e. < 2 µm) and numerous chromosomes (Leitch et al., 2010; Franklin de Melo et al., 1997). Our results are not conclusive about the ploidy of *X. viscosa*. The chromosome count and the percentage of the non-reference alleles do not allow a definition of the genome as hexaploid or octoploid. A deeper sequencing coverage is necessary to clarify this matter.

ABA plays a key role in a broad array of adaptive stress responses to environmental stimuli in plants. The application of exogenous ABA was shown to mimic dehydration responses (Bianchi et al., 1992; Greenwood and Stark, 2014), although ABA-dependent and ABA-independent pathways are known to operate in response to abiotic stresses (Yamaguchi-Shinozaki and Shinozaki, 2005).

Among the ABA-dependent pathways, the interplay of ABA with other endogenous signalling molecules acts as an integrator of

environmental cues (Arc et al., 2013). The crosstalk between ABA and ethylene for example, influences seed dormancy and germination, as well as stomatal closure (Arc et al., 2013). In *A. thaliana* seeds, ABA limits ethylene action by downregulating its biosynthesis and reducing ethylene sensitivity (Arc et al., 2013). In *X. viscosa*, ABA and ethylene induce the accumulation of *XVT8* (a dehydrin) transcripts in adult plants (Ndima et al., 2001) and transcripts and proteins in seedlings (both shoots and roots). We observed an over-representation of genes related to S-adenosylmethionine (an ethylene precursor) and ethylene biosynthesis in transcripts with the patterns "S↑,R↑" and "S→,R↑", suggesting that different stress signalling responses may be operating (Suppl Table 6.3). Moreover, some *X. viscosa* genes that accumulate transcripts in shoots (not in roots) and are homologs of genes that putatively delay senescence are highly similar to EINs (ETHYLENE INSENSITIVE) and ERSs (ETHYLENE RESPONSE SENSOR). EINs and ERSs act in ethylene signalling and mediation of cross-links between hormonal response pathways in plant responses to abiotic and biotic stresses (Arc et al., 2013). Taken together, these observations highlight the likely importance of a hormonal crosstalk in the induction of DT.

The role of ABA in growth inhibition is well documented. For example, exogenous ABA inhibits growth of germinated *A. thaliana* seeds (Costa et al., 2015; Maia et al., 2014), gemmalings and protonema of submerged liverworts (Akter et al., 2014; Hartung, 2010), and the algae *Coscinodiscus* (Hartung, 2010). Incubation of *X. viscosa* seedlings in ABA also inhibited growth. This inhibition is corroborated by transcriptomic evidence, as several GO categories related to growth were over-represented in the patterns "S↓,R↓" and "S↓,R→". Growth inhibition seems to be achieved through the down-regulation of genes related to different mechanisms, including cell expansion and separation, cell wall formation, shoot meristem size, vascular strand development in the leaf and control of leaf shape, size and symmetry.

It has been hypothesized that ABA signalling evolved in the plant kingdom because of the high selective pressure exerted by the temporal absence of water (Hauser et al., 2011). An evidence in favour of this hypothesis is the effect of exogenous ABA in decreasing the permeability of leaf bundle-sheath cells to water, creating an intraleaf barrier by

downregulating aquaporin activity (Shatil-Cohen et al., 2011). We observed this same effect in *X. viscosa* seedlings. The GO category *water transport* was over-represented in the pattern "S↓,R↓", with decreasing transcript abundance for putative aquaporin genes.

Several molecules, such as reactive oxygen species (ROS) and transcription factors (TFs), act as intracellular messengers and transmit specific aspects of ABA signalling (Hauser et al., 2011; Lindemose et al., 2013). ABA causes the production of ROS that down-regulate the activity of the Group A PP2C phosphatases. *A. thaliana* contains nine Group A PP2C genes, including *AHG1, AIP, HAB1* and *HAI*, while *P. patens* has only two (Sakata et al., 2009) and *X. viscosa* has five. It has been hypothesized that land plants developed increased numbers of Group A PP2C genes during their evolution to enable tissue- and organ-specific coordination of ABA signalling (Sakata et al., 2009). In *X. viscosa*, the Group A PP2Cs *HAI-1* and *HAI-2* accumulate transcripts in shoots, while *HAB1* declines in transcript abundance. ROS also activate Ca^{2+}-permeable channels that may activate calmodulin-binding transcription activators (CAMTAs) (Hauser et al., 2011). In shoots of *X. viscosa* seedlings, direct targets positively regulated by Ca^{2+} seem to be activated by ABA, but putative CAMTA genes were not significantly affected (Fig 6.4). On the other hand, in roots, a calmodulin and a CAMTA gene were repressed by incubation in ABA. Several TFs also function as mediators of ABA response. The TF MYB44 is induced by ABA treatment and its over-expression in transgenic *A. thaliana* plants enhances ABA sensitivity and drought tolerance (Fujita et al., 2011). WRKY TFs are considered early nodes in ABA-signalling (Lindemose et al., 2013). WRKY63 is one of the central components of the ABA-dependent gene regulatory network as it directly regulates *ABF*s (ABA responsive element binding factors), while WRKY2 regulates *ABI5*, *ABI3* and some LEA genes (Lindemose et al., 2013). Moreover, PYRLs and SnRKs (sucrose non-fermenting1-related protein kinases) are essential core components of the upstream network that regulates ABA-responsive processes (Arc et al., 2013). In *X. viscosa* seedlings, transcripts of *MYB44*, *WRKY63*, *WRKY2* and a number of PYRLs and SnRKs significantly accumulated in shoots upon ABA treatment, but not in roots (Fig 6.6). These differences in signal transduction may be an important factor to explain root sensitivity and shoot tolerance to fast

drying after ABA incubation.

LEA proteins comprise a large and heterogeneous group of proteins mainly expressed in seeds, although some are induced in vegetative tissues by ABA, dehydration, salt stress and low temperature (Tunnacliffe and Wise, 2007). In general, LEA proteins have been regarded as part of the cell protective apparatus against biotic and abiotic stress (Tunnacliffe and Wise, 2007). LEA protein encoding genes have been identified in a variety of species, from plants to invertebrates and microorganisms (Tunnacliffe and Wise, 2007). For example, 51 LEA protein encoding genes were identified in *A. thaliana* (Hundertmark et al., 2011), 29 in *Castanospermum australe* (Delahaie et al., 2013), 16 in *Medicago truncatula* (Chatelain et al., 2012) and 13 in *Z. mays* (Amara et al., 2012). In *X. viscosa*, 21 LEA-like proteins have been previously identified and two have been functionally characterized (Farrant et al., 2015). Here, we took a genome-wide approach to mine for a more complete set of LEA-like proteins. This analysis resulted in a set of 87 putative LEA protein encoding genes (Suppl Table 6.5). This seems to be the biggest set of LEA protein encoding genes reported for a single species, suggesting a higher number of LEA proteins in resurrection species than in non-resurrection species. Further analyses are needed to understand the function and genome organization of these proteins.

According to sequence analysis, 48% of these LEA genes represent two different classes: groups 6 and 8 (Hunault and Jaspard, 2010). Group 6 LEA proteins (Pfam PF02987) are characterized by a repeating motif of 11 amino acids and a very low proline content and are predicted to be at least 50% unfolded in the hydrated state (Amara et al., 2014; Jaspard et al., 2012). Expression analysis of group 6 LEA proteins show their accumulation in mature *A. thaliana* seeds and in response to ABA and stress conditions (Amara et al., 2014). Group 8 LEA proteins (Pfam PF03168) are characterized by their small size and high content of structured regions (Amara et al., 2014; Jaspard et al., 2012). Most of the LEA protein encoding genes identified in the genome of *X. viscosa* showed higher abundance of transcripts and proteins in shoots than in roots, reinforcing the ability to activate the transcription of these genes and accumulate LEA proteins as crucial for the onset of DT.

The ability of resurrection plants to avoid senescence during

dehydration is a key feature of DT (Griffiths et al., 2014). During dehydration, *X. viscosa* leaves selectively degrade chlorophyll-binding proteins and avoid Rubisco degradation (Christ et al., 2014). In accordance, one third of the *A. thaliana* genes that delay senescence and do not have a putative homolog in *X. viscosa* genome are related to Rubisco degradation (Ono et al., 2013). GO categories related to cell division were enriched in *X. viscosa* genes that putatively promote senescence and which showed a decrease in transcript abundance in shoots. Some genes in these categories are putative Cyclin-Ds, that act in modulating growth rate in response to environmental and developmental conditions (Cockcroft et al., 2000). Among the *X. viscosa* genes that putatively delay senescence and accumulate transcripts in shoots, there are homologs of *PUB43* and *PUB44* (PLANT U-BOX). *PUB43* and *PUB44* are thought to delay senescence and cell death by negatively regulating ABA levels through inhibition of ABA biosynthesis genes (Salt et al., 2011).

Much resurrection plant research has focused on understanding how these plants survive desiccation and how DT pathways are regulated. We have shown the importance of signal transduction in the ABA signalling pathway for DT (Fig 6.8). Furthermore, the cross talk between ABA and ethylene together with senescence prevention by avoidance of Rubisco degradation and modulation of growth rate might be key factors for the basic framework of the resurrection phenotype. Finally, the increased number of LEA protein encoding genes (compared to non-resurrection plants) and the ability to transcribe and accumulate LEA proteins are main players in the induction of DT.

DT is a complex phenomenon. The use of next generation high-throughput technologies is enabling us to better understand its complexity and deepen our knowledge on the main mechanisms controlling it. These mechanisms are powerful tools to explain the evolution of DT and to ultimately improve drought tolerance in sensitive crops.

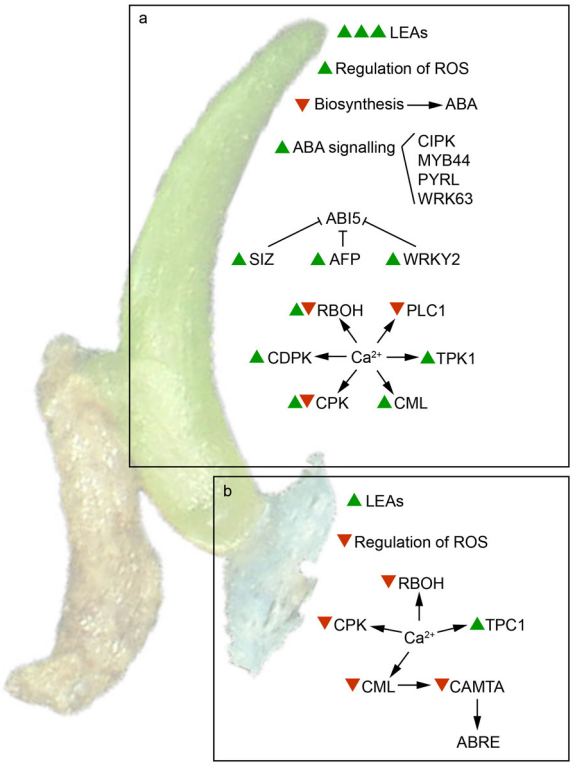

Fig 6.8 *Diagram of changes occurring in* a *shoots and* b *roots of* X. viscosa *seedlings in response to exogenous ABA. Only contrasting changes are represented. The number of late embryogenesis abundant (LEA) protein encoding genes with accumulating transcripts, is higher in shoots than in roots. Genes involved in the regulation of reactive oxygen species (ROS) are over-represented among the genes increasing their transcript abundance in shoots and decreasing it in roots. Genes involved in the biosynthesis of ABA showed a decline in transcript abundance only in shoots. Genes related to ABA signalling and negative regulators of* ABI5 *accumulated transcripts in shoots, but not in roots. A higher number of genes which are positively regulated by* Ca^{2+} *displayed a higher transcript abundance in shoots than in roots.* Green triangles *indicate increased and* red triangles *indicate decreased transcript abundance.*

Acknowledgements

This work was supported by the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Brazil). The authors thank Prof. Dr. Hans de Jong for the chromosome counting.

Supplementary Data

Supplementary Data can be downloaded by scanning or from: http://www.wageningenseedlab.nl/thesis/mcdcosta/SI/chapter6

Suppl Fig 6.1 Water content of *X. viscosa* seedlings during slow and fast drying. Slow drying was performed at 95% RH for 3 days, followed by 3 additional days at 32% RH. Fast drying was performed at 32% RH for 3 days. Data points are averages of 5 replicates of 20 seedlings. *Bars* represent standard error.

Suppl Fig 6.2 Typical picture used for chromosome count of *X. viscosa.*

Suppl Fig 6.3 Ploidy likelihood based on the alternate frequency of non-reference alleles.

Suppl Table 6.1 Primer sequences and expression profile of 12 genes measured by qPCR and RNA-Seq in shoots of *X. viscosa* seedlings before and after ABA treatment. The expression levels were normalised using 3 stably expressed genes from the transcriptome data and calculated with the qBase software (Hellemans et al., 2007).

Suppl Table 6.2 Desiccation tolerance in *X. viscosa* seedlings after 3 days of fast drying preceded or not by 3 days of incubation in solutions of ABA, PEG and combinations of ABA and PEG. Each data point represents the average of 3 replicates of at least 15 seedlings (±SE). Different *letters* indicate significant differences ($P \leq 0.05$) after one-way ANOVA with Tukey post-hoc test.

Suppl Table 6.3 Over-representation analysis of the differentially expressed genes comparing *X. viscosa* ABA-treated and -untreated shoots (S) and roots (R). Over-represented categories were determined using the plugin BiNGO (Maere et al., 2005) for Cytoscape. Redundant GO terms were removed using the online tool ReviGO (Supel et al., 2011) applying a cut-off of ≤ 0.05 for "Dispensability" value. ↓: reduced expression, ↑: increased expression, →: unchanged expression.

Suppl Table 6.4 List of *X. viscosa* homologs of proteins involved in ABA responses (based on Hauser et al., 2011).

Suppl Table 6.5 Putative LEA protein encoding genes of *X. viscosa* and their best homologs in the LEAPdb database. S: shoots, R: roots, ↓: reduced expression, ↑: increased expression, →: unchanged expression, #N/A: no data available.

Suppl Table 6.6 List of *X. viscosa* homologs of genes involved in leaf senescence in *A. thaliana* (Li et al., 2012). S: shoots, R: roots, ↓: reduced expression, ↑: increased expression, →: unchanged expression, #N/A: no data available.

Chapter
Seven

Key genes involved in desiccation tolerance and dormancy across life forms

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In preparation

Abstract

Certain organisms have the remarkable ability to survive severe dehydration (desiccation tolerance). Examples of these organisms can be found among insects, lichens, plants, rotifers, tardigrades, worms and yeasts. These naturally stress tolerant organisms may be a good source of genetic information to generate stress tolerant crops to face a future with predicted higher occurrence of drought episodes. By mining for key genes and mechanisms related to desiccation tolerance conserved across different species and life forms, unique candidate key genes may be identified. These genes could be useful in genetic engineering and in breeding programs aiming to improve abiotic stress tolerance in crops. Here we identify several of these putative key genes, shared among multiple organisms, encoding for proteins involved in protection, growth and energy metabolism. Mutating a selection of these genes in the model plant *Arabidopsis thaliana* resulted in clear desiccation tolerance- and other seed-associated phenotypes, such as dormancy, showing the efficiency and power of our approach and paving the way for the development of drought-stress tolerant crops. Our analysis supports a co-evolution of desiccation tolerance and dormancy by shared mechanisms that favour survival and adaptation to ever-changing environments with strong seasonal fluctuations.

Keywords

Re-establishment of desiccation tolerance, dormancy, ABI3, *Arabidopsis thaliana*

Introduction

Climate model simulations predict that by 2100 the climate will be several degrees warmer than it is now and precipitation will decrease in most tropical and mid-latitude regions, expanding the area of global dryland by ~10% and severely reducing yields for primary crops like corn, wheat and rice (Sherwood and Fu, 2014b). Considering the devastating impact of drought on global agriculture, feeding a world with an increasing population that may reach 9 billion by 2050 will be a challenge (Varshney et al., 2011). In this context, it is essential to breed for plant varieties which are optimally adapted to the changing environment and maximally stress tolerant (Varshney et al., 2011). Genes that confer stress tolerance can be mined from several sources, but particularly from organisms that are naturally able to survive variable and extreme conditions, such as those that are desiccation tolerant.

Desiccation tolerant organisms have the ability to resist severe drought by surviving drying to equilibrium below 0.1 g of water per gram dry weight with moderately dry air (50-70% relative humidity at 20-30˚C), and resume normal metabolism upon rehydration (Bewley et al., 1979). Desiccation tolerance (DT) was a key trait in ancient non-vascular plants (Oliver et al., 2005). However, most of the modern vascular plants have retained DT only in a few specialized structures, such as seed and pollen. Seeds of more than 90% of the modern angiosperms for which data are available can tolerate desiccation (Royal Botanic Gardens Kew, 2008). In these plants, desiccation tolerant seeds are considered necessary for the completion of their life cycle, increasing seed life span and aiding dispersal (Ramanjulu and Bartels, 2002). There is a small number of vascular plants (about 320 species), termed "resurrection plants" (Gaff, 1971) that are able to acquire DT in their vegetative tissues and to survive repeated cycles of desiccation and rehydration (Gaff, 1989; Farrant, 2000). Therefore, both desiccation tolerant seeds and resurrection plants are promising genetic resources for improving drought tolerance in crop species (Varshney et al., 2011).

As the majority of resurrection plants are seed plants, it has been hypothesized that the mechanism of DT in seeds is the likely source of genetic reprogramming for the evolution of all extant angiosperm

resurrection plants (Bewley and Oliver, 1992; Farrant and Moore, 2011; Oliver et al., 2000). However, recent evidence suggests that this reprogramming may have occurred earlier in the fern *Pteris vitatta* via desiccation tolerant spores on a desiccation sensitive plant (Ballesteros et al., 2012). In light of this hypothesis, several recent studies have focused on cellular mechanisms and gene expression patterns associated with DT in seeds and resurrection plants. However, few studies have addressed genetic traits that are conserved in both desiccation tolerant seeds and plants (Amara et al., 2013; Buitink et al., 2003; Farrant et al., 2012; Illing et al., 2005) and a molecular similarity between DT mechanisms in seeds and resurrection plants remains to be convincingly demonstrated (Farrant and Moore, 2011). Therefore, we have adopted a cross-species approach, using published gene expression data sets on re-induction of DT in germinated seeds and desiccation responses of resurrection plants, a lichen and a nematode to search for conserved DT genes. By doing this, a core set of genes was identified for which *Arabidopsis thaliana* T-DNA insertion mutants had a high incidence of DT and seed-associated phenotypes. Furthermore, the high incidence of dormancy-related genes among the identified core set supports the hypothesis of co-evolution of DT and dormancy as a strategy to survive fluctuating environments.

Materials and methods

Data set collection and detection of orthologs

Desiccation tolerance-related transcriptome profiling data were obtained for four plant species from available databases. The databases referred to experiments that monitored: (1) the transcriptional response of germinated desiccation sensitive *A. thaliana* seeds at the stage of radicle protrusion, to the re-induction of DT by polyethylene glycol (PEG, GEO Series accession number GSE64227) or by abscisic acid (ABA, GEO Series accession number GSE62876); the overlap between these two independent data sets was used for the comparison with the other sets; (2) the transcriptional responses of germinated *Medicago truncatula*

seeds with protruded radicle lengths of 2.7-2.9 mm to re-induction of DT (Terrasson et al., 2013); (3) the responses of *Craterostigma plantagineum* leaves to desiccation (desiccation for 15 days, reaching 5 % of relative water content, RWC) compared to fully hydrated leaves (Rodriguez et al., 2010); and (4) the responses of leaves of the monocot *Sporobolus stapfianus* to desiccation (0.5 g H_2O g^{-1} dw^{-1}) compared to fully hydrated leaves (GEO Series accession number GSE64900).

In the case of both *A. thaliana* data sets, Affymetrix ARAGene 1.1ST Arrays were used and linear modelling was applied with thresholds for absolute fold change of ≥ 1.0 (on a $\log_2$ scale) and ≤ 0.01 for *P*-value. For *M. truncatula*, Medtr_v1.0 12x135K arrays (synthesized by Roche NimbleGen) were used (Terrasson et al., 2013) with thresholds for absolute fold change of ≥ 1.0 (on a $\log_2$ scale) and ≤ 0.01 for *P*-value. Fold change values from the *C. plantagineum* data set were calculated based on expression values of all assembled transcripts provided by Rodriguez et al. (2010) and the absolute value for fold change was set as ≥ 2.0 (on a $\log_2$ scale). The *S. stapfianus* data set was derived from a custom oligoarray, 7x60mer probes per contig constructed from 454 sequencing of pooled cDNAs, synthesized and hybridized with single dye labelling by Roche NimbleGen (Madison, WI). The expression data was also analysed using linear modelling with thresholds for absolute fold change of ≥ 1.0 (on a $\log_2$ scale) and ≤ 0.01 for *P*-value.

C. plantagineum sequences were downloaded from NCBI (www.ncbi.nlm.nih.gov). CDS sequences of *M. truncatula* genes (version Mt3.5v3) were downloaded from LegumeIP (http://plantgrn.noble.org/LegumeIP/). *S. stapfianus* sequences were obtained from M. Oliver (unpublished). The sequences were compared against the whole *A. thaliana* genome using BLASTP in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To test the robustness of our analysis, we also compared the sequences of *C. plantagineum* and *S. stapfianus* against the whole *M. truncatula* genome using BLASTP in LegumeIP (http://plantgrn.noble.org/LegumeIP/blast.do), resulting in no appreciable differences.

Orthologs were defined as hits with lowest Expect value (E-value), with a threshold of ≤ 10^{-20}. Multiple hits were considered orthologs when the difference between their E-values and the lowest hit's E-value was smaller than 10^{-10}. To search for orthologs, the sequence of each

transcript was compared the whole genome sequence of *A. thaliana* (TAIR10) using online tools available on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The analysis was conducted for accumulating and declining transcripts separately, so only genes with similar transcript expression profiles across different species would be selected.

Phenotypic characterization

Genes were selected for phenotypic characterization because they were seed specific in *A. thaliana* seeds (when the ratio between their maximum expression value in seeds and the maximum expression value in leaves or roots was higher than 2). Expression values were obtained from the Expression Browser (Toufighi et al., 2005) and belonged to at least one of the following GO categories: *response to stress, response to stimulus* or *seed development*. T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Centre (Suppl Table 4.1). T-DNA insertions were confirmed using PCR. Seeds of plants homozygous for the mutations and wild-type (WT, Col-0) seeds were grown on Rockwool, in a climate cell (20˚C day, 18˚C night), with 16 h of light (35 W m^{-2}), and watered with Hyponex nutrient solution (1g l^{-1}, http://www.hyponex.co.jp). Seeds obtained from these lines were bulk harvested in three replicates of at least two plants and were phenotyped to evaluate the ability to re-establish DT according to the protocol described by Maia et al. (2011) for re-induction of DT in germinated seeds at appearance of first root hairs (stage IV in Maia et al., 2011). Averages were calculated and a Student's t-test was performed.

The degree of seed dormancy was evaluated as the number of Days of Seed Dry Storage required to reach 50% germination (DSDS50) according to Alonso-Blanco et al. (2003). Seed longevity was estimated based on the performance of the seeds in a germination test after undergoing accelerated aging. In order to accelerate aging, seeds were stored for six days in a closed container above a saturated NaCl solution (80% relative humidity) at 40˚C in the dark. Seed vigour was assessed as the ability of the seeds to germinate at high temperature (at 30˚C) or on NaCl (125 mM). The germination experiments and germination

scoring were performed as described by Joosen et al. (2012). All germination tests were performed in a fully randomized setup. For each measurement, we used three replicates of seeds harvested from at least two plants. To determine dry seed size, close-up photographs from 200 to 300 seeds were analysed by ImageJ as described by Joosen et al. (2010).

Motif and GO category analysis

To search for the occurrence of *cis*-acting elements potentially involved in regulating the expression of the shortlisted genes, the web-based tool Athena (*Arabidopsis thaliana* expression network analysis) was used (O'Connor et al., 2005). The promoter regions were searched for any common motifs located within a 1 kb region upstream of the translational start site.

GeneTrail (http://genetrail.bioinf.uni-sb.de/index.php, Keller et al., 2008) was used to determine enrichments of functional annotations among the shortlisted genes. The tests were performed using all Gene Ontology (GO) categories and the *P*-value was adjusted according to the Bonferroni method. Enriched GO terms for Biological Process were further processed using the ReviGO online tool (http://revigo.irb.hr/) to remove redundant terms based on a cut-off of "dispensability" < 0.1 (Supek et al., 2011).

The frequency of *Caenorhabditis elegans* and *Cladonia rangiferina* putative orthologous genes, members of the ABI3 regulon, dormancy-related genes and evolutionary old genes in the 260 shortlisted genes was tested against a background frequency generated using 1,000 randomized samples of 260 *A. thaliana* genes from a set of 27,981 (from *A. thaliana* microarrays).

Results

Data sets and detection of orthologs between different species and organs

Our primary objective was to identify a core set of genes involved in the response to desiccation that is conserved across species and different organs (i.e. seeds and leaves). To do so, publicly available gene expression data were obtained for four species (*A. thaliana, M. truncatula, C. plantagineum* and *S. stapfianus*), from five separate studies employing different experimental conditions.

A differential expressed gene list was obtained for each dataset. Due to differences in experimental design and size of the data sets, the defined threshold of absolute fold change was adjusted as indicated in Table 7.1. The overall number of differentially expressed genes (DEGs) retained for each experiment is shown in Table 7.1. In the case of *A. thaliana*, two data sets were compared and 1,993 DEGs representing the overlap between them were used for further analysis. To compare all the data sets, it was necessary to identify orthologs among the tested species. We identified 9,625 *A. thaliana* genes as orthologs of DEGs in the other sets, thus for 67.95% of the analysed genes a probable *A. thaliana* ortholog could be found.

Table 7.1 *Datasets size, defined threshold of absolute fold change and number of DEGs in each of the used data sets*

Species	Data set size	Absolute fold change*	Accumulated transcripts	Declined transcripts
Arabidopsis thaliana - ABA	27,981	1.0	1,593	1,933
Arabidopsis thaliana - PEG	27,981	1.0	1,231	2,169
Overlap between *Arabidopsis thaliana* data sets	-	-	824	1,169
Medicago truncatula	10,289	1.0	2,913	5,803
Craterostigma plantagineum	15,093	2.0	2,278	2119
Sporobolus stapfianus	50,690**	1.0	440	611

** Values are presented on a $\log_2$ scale*
*** Contigs*

Identification of a core set of DT genes conserved across species

Transcripts of 12 genes (2 up-regulated, 10 down-regulated) were present in all data sets (Fig 7.1, Suppl Table 7.2). Transcripts of one gene (*EARLY-RESPONSIVE TO DEHYDRATION 7,* AT2G17840) were

present in both increasing and decreasing shortlists because this gene was a putative ortholog of accumulating and declining gene transcripts of both *M. truncatula* and *C. plantagineum*. The two accumulating transcripts present in all data sets are an NAD(P)-linked oxidoreductase superfamily protein (AT5G01670) and *LEA 14* (AT1G01470). The ten genes represented by declining transcript levels in all four data sets were two actins (AT3G12110 and AT3G53750), *ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT 2* (*APEE2,* AT5G46110), *BETA-XYLOSIDASE 2* (*BXL2,* AT1G02640), *GAMMA TONOPLAST INTRINSIC PROTEIN* (*GAMMA-TIP,* AT2G36830), GDSL-like lipase / Acylhydrolase superfamily protein (AT1G28580), *GLYCOLATE OXIDASE 1* (*GOX1,* AT3G14420), *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1,* AT5G46110), *PLASMA MEMBRANE INTRINSIC PROTEIN 1C* (*PIP1C,* AT1G01620) and *PURPLE ACID PHOSPHATASE 1* (*PAP1,* AT2G16430).

By comparing all data sets, we found that 260 *A. thaliana* orthologs were shared by at least three species (Fig 7.1, Suppl Table 7.2). Of these transcripts, 77 increased and 184 declined in abundance. Examination of the genes represented by accumulating transcripts revealed five major GO enriched categories: *lipid storage, multicellular organismal process, seed development, reproduction* and *response to temperature stimulus* (Suppl Table 7.3). In general, shortlisted genes that are represented by declining transcripts are enriched in the GO categories related to carbon fixation (Calvin-Benson cycle), photosynthesis, phenylpropanoid biosynthetic process (toward compounds that act on peroxidases as acceptors), and starch and sucrose metabolic process (Suppl Table 7.3).

Analysis of enrichment of *cis*-acting promoter elements in the core gene set using the Athena package (O'Connor et al., 2005) (Table 7.2), showed that among the genes represented by accumulating transcripts, the enriched motifs were related to elements found in genes that respond to ABA (ABRE binding site motif, ABRE-like binding site motif and ACGTABREMOTIFA2OSEM), light regulation (CACGTGMOTIF), stress (DRE core motif), circadian clock (EveningElement promoter motif) and gibberellin (GADOWNAT). Among the genes represented by declining transcripts, the enriched motifs were ABRE binding site (related to response to stress) and TATA-box (related to control of gene transcription).

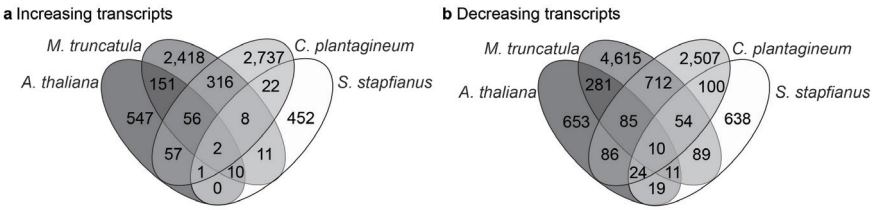

Fig 7.1 *Venn diagrams of orthologs in the desiccation tolerance data sets. The analysis was conducted for* a *increasing and* b *decreasing transcripts separately.*

Table 7.2 *Enriched promoter motifs in desiccation tolerance core gene set.*

Enriched Transcription Factor/Motif	P-value
Up-regulated	
ABRE binding site motif	$< 10^{-5}$
ABRE-like binding site motif	$< 10^{-10}$
ACGTABREMOTIFA2OSEM	$< 10^{-10}$
CACGTGMOTIF	$< 10^{-10}$
DRE core motif	$< 10^{-7}$
EveningElement promoter motif	$< 10^{-5}$
GADOWNAT	$< 10^{-9}$
Down-regulated	
ABRE binding site motif	$< 10^{-5}$
TATA-box motif	$< 10^{-10}$

Comparison of the DT plant core set with other organisms

Several species of nematodes have been reported to be desiccation-tolerant and, like seeds and resurrection plants, these accumulate LEA proteins, antioxidants, and non-reducing sugars (Browne et al., 2002). To investigate whether any of the core 260 shortlisted genes identified in this study have putative orthologs in nematodes, we used publicly available gene expression data from a study involving the desiccation tolerant nematode *C. elegans* in the "dauer" larva stage before and after preconditioning (Erkut et al., 2013). Dauer larvae of *C. elegans* are desiccation sensitive, but a preconditioning treatment makes them desiccation tolerant. Sequences of genes represented by significantly accumulating and declining transcripts following preconditioning (Erkut et al., 2013) were compared to the genome sequence of *A. thaliana* using NCBI Blast tools. Putative orthologs were selected, resulting in 15% of the *C. elegans* gene transcript sequences with identifiable orthologs in the *A. thaliana* genome (Suppl Table 7.4). We found high similarity

scores for 34 of the core 260 genes among DT-related *C. elegans* genes (Fig 7.2, Suppl Table 7.4), a number significantly higher (*P*-value < 0.01) than expected by random selection. The seven genes that accumulate transcripts during desiccation and shared by our core shortlist and the data set for *C. elegans* are involved in several processes, e.g., *cell protection* and *protein amino acid phosphorylation*. The 27 genes that exhibit transcript decline during desiccation and shared by our shortlist and the data set for *C. elegans* are primarily related to cell growth, transport and protein kinase activity.

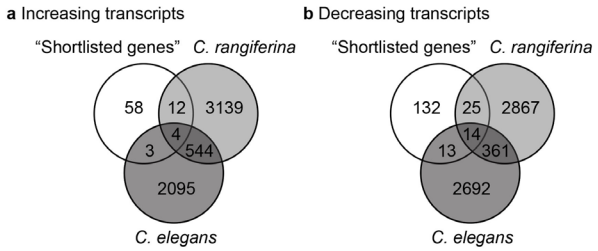

Fig 7.2 *Venn diagrams of genes in common between the core 260 shortlisted genes in this study and DT-related* Caenohabditis elegans *and* Cladonia rangiferina *genes. The analysis was conducted for* a *increasing and* b *decreasing transcripts separately.*

Most lichens are highly desiccation tolerant and can withstand drying to water contents less than 5% within a few minutes (Junttila et al., 2013). Antioxidants such as glutathione and free radical scavenging enzymes such as superoxide dismutase, catalase, and several peroxidases were demonstrated to be critically important for their survival of desiccation. Sequences of DEGs in response to desiccation of the grey reindeer lichen *C. rangiferina* (Junttila et al., 2013) were compared to the genome sequence of *A. thaliana*. Putative orthologs were selected, resulting in 49% of the *C. rangiferina* genes with clearly identifiable orthologs in the *A. thaliana* genome (Suppl Table 7.4). We found high similarity scores for 55 of the core 260 shortlisted genes among DT-related genes from *C. rangiferina* (Fig 7.2, Suppl Table 7.4), a number significantly higher (*P*-value < 0.01) than expected by random selection. The 16 genes that accumulate transcripts during desiccation and shared by the core gene set and the data set for *C. rangiferina* are primarily related to response to stresses or are involved in protein metabolism. Most genes that exhibit

transcript decline during desiccation and shared by the core shortlist and the data set for *C. rangiferina* are related to biosynthesis of amino acids, cytochrome P450, lipid metabolism and transport. From the 55 genes with high similarity scores, 18 (4 accumulating and 14 declining transcripts) had also high similarity with DT related *C. elegans* genes (Suppl Table 7.4), a number significantly higher (*P*-value < 0.01) than expected by random selection.

Characterization of A. thaliana mutant lines

Mature seeds of *A. thaliana* are desiccation tolerant and, upon germination, progressively become desiccation sensitive. However, DT can be rescued during the early stages of germination by an osmotic treatment with PEG (Maia et al., 2011) or ABA (Chapter 3). This re-induction of DT in germinated seeds allows the separation of mechanisms related to the acquisition of DT and those involved in concomitant seedling developmental processes (Buitink et al., 2003; Dekkers et al., 2015). Maia et al. (2011) described the application of this model in germinated *A. thaliana* seeds in four clearly distinct stages: (1) seeds at testa rupture, (2) at radical protrusion, (3) with primary roots of approximately 0.3 mm length, and (4) at appearance of the first root hairs. To investigate the role of some of the identified conserved genes putatively involved in DT, we focused on T-DNA mutant lines and their behaviour at the appearance of the first root hairs, when WT seeds are less competent in re-establishing DT and thus allowing a clear distinction between mutant lines. Thirteen genes from the core 260 shortlisted gene set were chosen for phenotypic characterization in T-DNA mutant lines (Table 7.3) based on their seed-specific expression (detailed in Materials and methods). Of the 13 mutant lines, three displayed a reduced ability to re-induce DT compared to WT (*RCI2H*, *LEA 5-1* and *PCK1*, Fig 7.3). The remainder showed no phenotypic DT-related differences to WT (Suppl Table 7.5).

As DT is a multigenic and complex trait, the mutation of one gene is unlikely to entirely reduce survival of the desiccated state (Delahaie et al., 2013; Hundertmark et al., 2011). We thus tested the performance of the T-DNA mutant lines in relation to seed vigour (germination under

Table 7.3 *Genes chosen for phenotypic characterization by T-DNA insertion mutants.*

AGI	Annotation	DT Inc/Dec	DT re-induction	Seed vigour		Seed dormancy	Seed longevity
				High temperature	Salt	DSDS50	Accelerated aging
AT1G48130	1-CYSTEINE PEROXIREDOXIN (PER1)	Inc					
AT1G52690	LEA7	Inc				↑	
AT1G72100	LEA domain-containing protein	Inc					
AT2G38905	RARE COLD-INDUCIBLE GENE 2H (RCI2H)	Inc	↓↓			↑	↑
AT2G47180	GALACTINOL SYNTHASE 1 (GolS1)	Inc			↓↓		
AT3G10020	Unknown protein	Inc			↓↓	↑↑	
AT3G22490	LEA5-1	Inc	↓↓	↓	↓↓		
AT4G02280	SUCROSE SYNTHASE 3 (SUS3)	Inc					
AT4G37870	PHOSPHOENOLPYRUVATE CARBOXYKINASE (PCK1)	Dec	↓	↓↓	↓	↑↑	↓
AT5G01670	NAD(P)-linked oxidoreductase superfamily protein	Inc					
AT5G06760	LEA1	Inc					
AT5G07330	Unknown protein	Inc					
AT5G13170	SENESCENCE ASSOCIATED GENE 29 (SAG29)	Dec				↑	

Columns provide annotation, whether they increase (Inc) or decrease (Dec) in expression during desiccation, and phenotyping results. Arrows indicate significant increase (↑) or decrease (↓) as compared to the wild type at $P \leq 0.05$ for single and $P \leq 0.01$ for double arrows.

conditions of high temperature or salt exposure), dormancy, longevity (germination after accelerated ageing), and mature air-dry seed size (Table 7.3). None of the lines produced seeds with a statistically different dry seed size in relation to WT. Four lines (*GolS1,* AT3G10020*, LEA5-1* and *PCK1*) had reduced seed vigour compared to WT and five lines (*LEA7, RCI2H,* AT3G10020*, PCK1* and *SAG29*) had increased dormancy. Genes disrupted in these lines are functionally involved in primary metabolism or stress tolerance. Seeds of one line (*RCI2H*) had reduced seed longevity.

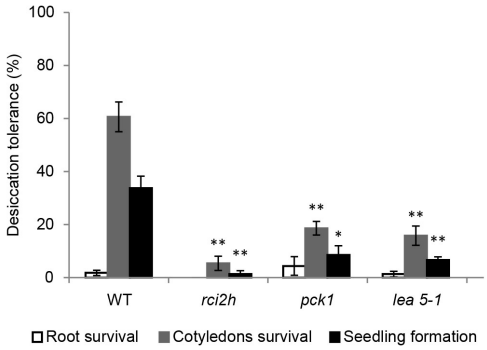

Fig 7.3 *Re-establishment of desiccation tolerance in germinated* A. thaliana *seeds of wild-type (WT) and T-DNA insertion lines scored as survival of primary root and cotyledons survival, and seedling formation.* Error bars *represent standard error.* Asterisks *indicate statistical significant differences compared to the WT at* $P \leq 0.05$ *for single and* $P \leq 0.01$ *for double asterisks. Experiments were conducted in three biological replicates of seeds bulk-harvested from at least two plants.*

Discussion

Desiccation tolerant life forms appear to utilize similar types of mechanisms to cope with the stresses imposed by the loss of water, suggesting DT to be an ancestral character (Alpert, 2006). Indeed, in plants, it has been proposed that DT of vegetative tissues was primitively present in bryophytes, lost in the evolution of tracheophytes and re-evolved multiple times (at least thirteen) in the history of angiosperms, mostly within herbaceous lineages (Alpert, 2006; Gaff and Oliver, 2013; Oliver et al., 2000, 2005). As DT was retained in specialized structures such as seeds and pollen in almost all angiosperms (Farrant and

Moore, 2011; Illing et al., 2005; Oliver et al., 2005, 2000), it is likely that DT genes remained present in the plant genomes and that the re-evolution of DT in vegetative tissues of higher plants arose by changes in regulation of gene expression rather than in the emergence of "new" genes (Gaff and Oliver, 2013). By comparing sets of DT-related genes in four different plant species, we were able to identify 260 genes within the *A. thaliana* genome with orthologs in at least two of the other species that are likely to be those genes retained for DT during the evolution of land plants. 44% of these genes were shown to be evolutionary old (pre-embryogenesis) genes according to the developmental hourglass model, a number significantly higher (*P*-value < 0.01) than expected by random selection (Quint et al., 2012). Comparisons of the core 260 shortlisted genes with DT-related genes in the nematode *C. elegans* and the lichen *C. rangiferina* suggest commonality among some of the main mechanisms involved in DT, such as formation of intracellular glass, maintenance of cell homeostasis, accumulation of protective sugars, LEA proteins and antioxidants, protection against mechanical stress, maintenance of cell skeleton integrity and cytochrome P450-family members (Browne et al., 2002; Farrant, 2000; Oliver et al., 2000).

Within the core 260 shortlisted genes, 18 genes have high similarity scores with DT-related genes in the nematode *C. elegans* and the lichen *C. rangiferina*. Several of these 18 genes, such as actin, aldehyde reductase, ferritin, glutamine synthetase and heat shock protein 70 (HSP70), have been reported to be conserved in plants and animals (Kumada et al., 1993; Mayer, 2013; Oberschall et al., 2000; Proudhon et al., 1996), confirming the power of our approach. Now, we show that the conservation of these genes is related to the evolution of DT, as their products help cells to minimize the damage from reactive oxygen species and to maintain cellular integrity during dehydration (Chen et al., 2005; Oberschall et al., 2000; Wang et al., 2009).

Of the 13 T-DNA mutant lines, three (*RCI2H*, *PCK1*, and *LEA 5-1*) exhibited a statistically significant difference in the re-acquisition of DT in germinated seeds compared to WT (Fig 7.3). *RCI2H* is a putative amino acid transporter, functioning in membrane potential homeostasis (Medina et al., 2007). Seeds of plants mutated in this gene displayed reduced re-acquisition of DT after germination, presumably

the result of disturbed membrane homeostasis. *PCK1* encodes a phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.49) shown to be down-regulated upon desiccation in several species, including *Xerophyta humilis* (Collett et al., 2004), *C. plantagineum* (Rodriguez et al., 2010) and *M. truncatula* (Terrasson et al., 2013). Germinated seeds of the T-DNA mutant line for this gene had a reduced capacity to re-establish DT, possessed deeper dormancy and an increased sensitivity to salt and high temperatures during germination compared to WT. We speculate that this is a consequence of the essential role of PCK1 in the mobilization of storage lipids and proteins via gluconeogenesis upon germination (Rylott et al., 2003). *In vitro* studies have demonstrated the involvement of PM25, a homolog of the seed maturation protein LEA5-1, in enzyme protection, anti-aggregation against thermo-mechanical stress and water binding (Boucher et al., 2010). A proteomic analysis of LEA proteins demonstrated the accumulation of the LEA5-1 ortholog in *M. truncatula* upon the acquisition of DT (Chatelain et al., 2012). Our results reinforce the importance of LEA5-1 in DT, as seeds mutated for this protein exhibit a reduced ability to re-establish DT.

We also evaluated the performance of the 13 mutant lines in relation to other seed-related traits (Table 7.3). Five lines showed increased dormancy compared to the WT. This observation led us to search for dormancy-related genes among the core 260 genes. Forty of these genes are differentially expressed comparing dormant states to after-ripened states in *A. thaliana* (Cadman et al., 2006). Additionally, around 6% of DT-related genes in *C. elegans* are also related to the exit from the dauer stage, a dormant-like developmental stage (Sinha et al., 2012). It may be argued that the development of desiccation-tolerant life forms, such as seeds, pollen and some larvae, must have been accompanied by the acquisition of dormancy or a dormancy-like stage. Terrestrial environments impose periods of stress through both intermittent and seasonal variation in climate. If water availability was the only requirement for survival, developing seedlings and larvae would often be subject to adverse conditions resulting in poor survival. Dormancy mechanisms evolved to reduce this risk. There is evidence of a higher incidence of dormancy in plants that have evolved in temperate areas and/or where seasonality is marked compared to those indigenous to

equatorial tropical rainforests (Baskin and Baskin, 2014). We speculate that a co-evolution of DT and dormancy by shared mechanisms would favour survival and adaptation to changing environments, especially in areas with strong seasonal fluctuations (Dekkers et al., 2015).

It is likely that the re-evolution of DT in vegetative tissues was achieved primarily by changes in gene expression (Gaff and Oliver, 2013). In the current study, we investigated the presence of genes whose expression is under the control of the seed-specific transcription factor of the B3 family, ABI3, within our data set. ABI3 is activated by ABA during water-deficit stress (Delahaie et al., 2013; Khandelwal et al., 2010) and seeds mutated in this gene have a reduced capacity to re-establish DT during early germination (Maia et al., 2014). The genome of the moss *Physcomitrella patens* contains four *ABI3*-like genes with homologs in liverworts (*Riccia fluitans* and *Marchantia polymorpha*), suggesting an ancestral character of gene networks involved in DT (Rensing et al., 2008). ABI3 transcriptional targets in *A. thaliana* have been identified and some were proposed to have a protective role during desiccation: small heat shock proteins, antioxidant functions, LEAs and oleosins (Mönke et al., 2012). Of our 260 shortlisted genes, 17 belong to the ABI3 regulon, which comprises 98 genes, a number significantly higher (P-value $\leq$ 0.01) than expected by random selection. Nine are either antioxidants (*AtPER1*), LEAs (AT1G72100, AT3G53040, *AtECP63*, *LEA4-5*, *LEA5-1*), or oleosins (*OLEO1, OLEO3* and AT3G01570). All three genes that exhibit a DT re-induction phenotype are members of the ABI3 regulon. Taken together, this suggests that ABA signalling via the ABI3 pathway is highly conserved in the establishment of DT, ranging from that typified in ancient lineages such as in the bryophytes, to the seeds of angiosperms. With the establishment of DT in seeds from the ancient form of vegetative DT, modifications of this signalling route may have occurred to accommodate a partially independent control of dormancy during seed development.

In summary, organisms evolved several mechanisms to deal with environmental stresses and to maximize survival of their species over time and these adaptive abilities led to the colonization of all of the diverse environments on Earth. Some mechanisms, such as those facilitating DT, were developed early in the history of life and were retained or lost and re-

acquired by different lineages during diversification. By adopting a cross-species approach, we demonstrated that DT mechanisms are encoded by genes that share high sequence similarity across very diverse species and life forms. Moreover, in some instances, the disruption of only one of these genes can impact the ability to achieve DT. Genes mined from this kind of analysis may prove fruitful candidates for efforts to improve abiotic stress tolerance in crop species.

Acknowledgements

This work was supported by the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Brazil).

Supplementary Data

Supplementary Data can be downloaded by scanning  or from http://www.wageningenseedlab.nl/thesis/mcdcosta/SI/chapter7

Suppl Table 7.1 T-DNA insertion mutants.

Suppl Table 7.2 The 260 *A. thaliana* genes found to be orthologs of DT-related genes in at least three of the analysed species: (At) *A. thaliana*, (Mt) *M. truncatula*, (Cp) *C. plantagineum*, (Ss) *S. stapfianus.*

Suppl Table 7.3 Summary of significant GO categories in the core 260 genes. *P*-values were adjusted according to Bonferroni. Enrichment was calculated as a ratio between the numbers of observed and expected genes (between parentheses, respectively).

Suppl Table 7.4 Genes in common between the core 260 shortlisted genes identified in the present study and DT-related genes of *Caenohabditis elegans* and *Cladonia rangiferina*. In bold, *Arabidopsis thaliana* genes that had high similarity with genes of both *C. elegans* and *C. rangiferina.*

Suppl Table 7.5 Phenotypic characterization of T-DNA insertion mutants.

Chapter
Eight

General Discussion

Desiccation tolerance – solutions for a dryer future

Climate change is widely recognized as the major environmental problem the world is facing, with negative impacts on crop production (Deikman et al., 2012; Yang et al., 2010). The projection of increased global demand for crop calories by 100% by 2050 makes it crucial to develop varieties that can yield well under harsh environments, which is critical for the prevention of food shortages (Deikman et al., 2012; Tilman et al., 2011; Varshney et al., 2011). These improvements can only be achieved through a combination of breeding and biotechnology (Deikman et al., 2012).

In face of these challenges, studies of desiccation tolerance (DT) emerged as a promising source of genetic information for plant breeders. Naturally stress tolerant organisms and life forms, such as resurrection plants and orthodox seeds, evolved a complex set of mechanisms to respond timely to desiccation. The responses of orthodox seeds can be seen already during development, when they undergo a genetically programmed desiccation phase. During this phase, the combination of DT, dormancy and longevity is developed, which ensures survival until meeting the appropriate conditions for seed germination and seedling establishment. Resurrection plants sense water shortage in the environment and activate a series of protective and adaptive responses, attempting to avoid desiccation- and rehydration-associated lethal damage. Different aspects of DT are well characterised, but others remain to be elucidated.

The work presented in this thesis focuses on the ability to (re-)induce DT in plant species at very specific desiccation sensitive developmental stages. The ultimate goal is to understand better the dynamics of DT and its ecological meaning and to use this information to mine for new candidate genes and pathways for drought-improvement in crops.

The ability to re-acquire desiccation tolerance

The ability of germinated orthodox seeds to re-acquire DT after

germination has been studied by several groups (**Chapter 2**). It relies on the activation of protective and adaptive mechanisms. A temporal analysis of the re-acquisition of DT in germinated Arabidopsis thaliana seeds by ABA treatment revealed that early events promote initial protection and growth arrest, while late events promote stress adaptation and contribute to survival in the dry state (**Chapter 3**).

From an ecological point of view, it is likely that DT acts in combination with dormancy to reduce the vulnerability of the stages between germinating seeds and established seedlings (**Chapters 2** and **7**). Dormancy ensures that germination does not occur until the conditions are favourable for seedling establishment. Yet, in the case of an unexpected water shortage during the first stages after germination, most seeds are able to put growth on hold, re-acquire DT and remain in the anhydrobiotic state until water is available again. After a certain developmental stage, seeds lose the possibility to re-acquire DT. For germinated *A. thaliana* seeds, this moment coincides with the appearance of the first root hairs. For germinated seeds of rice (*Oryza sativa*) (**Chapter 4**) and *Sesbania virgata* (**Chapter 5**), the ability to re-acquire DT is lost gradually following radicle protrusion and growth. In all cases, this ability is completely lost when seeds are able to anchor to the soil and actively take up water from it. This loss was also associated with the progression of the cell cycle towards cell division (**Chapter 4**), as growth reduction enhances abiotic stress tolerance and a higher DNA content increases stress sensitivity (Faria et al., 2005; Tang et al., 2012).

Treatments used to re-induce DT in germinated *A. thaliana* seeds involve incubation in an osmoticum and/or in abscisic acid (ABA) (Maia et al., 2011, 2014). Both treatments lead to re-induction of DT by partially overlapping mechanisms, since the osmotic treatment activates ABA-dependent and ABA-independent pathways (**Chapter 3**). Transcripts of genes related to seed development accumulate in response to both treatments and genes related to photosynthesis display a decline of transcript abundance.

Interestingly, an osmotic treatment and an ABA treatment seem to have different efficiencies in re-inducing DT in different species. Germinated *A. thaliana* seeds re-acquire DT faster by an ABA treatment than by an osmotic treatment (**Chapter 3**). Only an osmotic treatment

is effective for re-inducing DT in germinated seeds of rice (**Chapter 4**) and *S. virgata* (**Chapter 5**), as ABA alone does not stop radicle growth in these species. In seedlings of *Xerophyta viscosa*, osmotic and ABA treatments were equally effective in inducing DT; however, osmotic solutions produced a chlorotic phenotype and lower radicle survival (**Chapter 6**). It is likely that, in certain species, the osmotic treatment is needed to hold back growth until enough ABA has accumulated and acted, while in others, exogenous ABA accumulates and acts fast enough without the aid of the osmoticum.

DT is a prerequisite for seeds to acquire longevity, although desiccation tolerant seeds are not necessarily long-lived (Verdier et al., 2013). Orthodox seeds acquire DT and longevity during the maturation phase, before they enter a dormant or quiescent state in which they can remain for variable periods of time (Ooms et al., 1993; Toldi et al., 2009). During re-acquisition of DT by germinated seeds, longevity seems to be re-acquired later than DT (**Chapter 3**), likely to ensure the timely response of stress-related genes. However, this re-acquired longevity may be short. Germinated *S. virgata* seeds that did not lose DT could only be stored for short periods of time (up to 3 months at 4˚C) (**Chapter 5**) and a treatment to re-establish DT did not improve longevity. Uncoupling DT and longevity opens a series of possibilities to study both traits independently, with expected positive outcomes especially for *ex situ* conservation of short-lived seeds.

Desiccation vs drought

All desiccation tolerant plants are also drought tolerant, but the opposite is not true. Desiccation tolerant plants survive drying to below 0.1 g H_2O g^{-1} dry mass (Moore et al., 2008), while the drying limit tolerated by the drought tolerant ones is approximately 0.25-0.33 g H_2O g^{-1} dry mass (Hoekstra, 2002). Moreover, desiccation tolerant plants survive dry periods of several months (5-11 months) (Tuba and Lichtenthaler, 2011), while drought tolerance ensures survival for much shorter periods (hours to a day) (Kim et al., 2012). When plants sense

water scarcity in the soil, chemical signals travelling from the roots to the shoots induce changes in xylem conductance (Chaves et al., 2003). These changes are sensed by the leaves, causing stomata closure to prevent the risk of the plant losing its water transport capability (Chaves et al., 2003). Then, changes in osmotic potential optimize water retention. If drought becomes more severe, cell wall adjustments, growth reduction, antioxidant accumulation and inhibition of photosynthesis are observed (Harb et al., 2010). If drought persists, drought tolerant plants die, while desiccation tolerant plants activate efficient mechanisms for protection of cellular integrity and repair.

Various cultivars and genotypes of crop species display varying degrees of drought tolerance in their vegetative tissues, usually at the expense of crop yield. Cultivars combining improved drought resistance with yield potential under favourable conditions are, hence, the most promising for increasing productivity in drought-prone areas (Serraj et al., 2009). In this direction, efforts to dissect drought resistance by identification and characterization of component traits have had limited success especially because the timing and intensity of the drought are critical for these traits to be effective (Serraj et al., 2009). It is likely that this success can be improved with the use of traits that are effective in different life phases, for example, seed, seedling and adult plant.

An attempt to understand the relation between drought tolerance in adult plants and DT in germinated seeds is presented in **Chapter 4**. Rice seeds from cultivars differing in drought tolerance were used as experimental model. The results indicate that intrinsic mechanisms of drought tolerance in adult plants, such as growth control, are part of the mechanisms used by seeds to cope with water-deficit stress. More studies are necessary to understand the nature of these molecular mechanisms, as they can be the bridge between vegetative and seed DT and central for making progress in the field of crop improvement.

Exogenous applications of ABA have been shown to induce ABA-dependent drought-stress responses (Shinozaki and Yamaguchi-Shinozaki, 2007; Sreenivasulu et al., 2012). Accordingly, a large overlap was observed between genes that respond to exogenous ABA in germinated *A. thaliana* seeds and to drought in adult plants of the same species (**Chapter 3**). In general, genes of which transcript abundance

increased in response to drought responded relatively early to exogenous ABA. Moreover, transcriptome and proteome analysis of shoots and roots of *X. viscosa* subjected to exogenous ABA application suggests that differences in signal transduction may be an important factor to explain the differential responses of the different organs (**Chapter 6**).

New genes for crop improvement

A major hurdle for crop improvement programs faced by plant breeders is a limited gene pool of domesticated crop species (Deikman et al., 2012; Eldakak et al., 2013). Therefore, new candidate genes might prove useful for breeders. In this sense, conserved DT genes in the core of a common repertoire of responses to abiotic stresses should be good candidates. One possible approach to find these genes is to look into DT-transcriptomes of different species (**Chapter 7**). Accordingly, a comparison of four DT-transcriptomes of different plant species (two germinated orthodox seeds and two resurrection plants) resulted in a shortlist of 260 core genes. These genes are mainly involved in lipid storage, response to temperature stimulus, seed development, repression of primary metabolism and photosynthesis, and inhibition of water transport.

When *A. thaliana* mutant lines for a subset of these genes were phenotyped for seed-related traits, a number of lines showed decreased seed vigour (lower germination at high temperature or in salt solution) and increased seed dormancy compared to the wild type. These results confirmed the power of this approach in detecting genes from the core repertoire of responses to abiotic stresses.

Moreover, genes under the control of ABI3 were over-represented in the core 260. ABI3 is activated by ABA during water deficit stress (Delahaie et al., 2013; Khandelwal et al., 2010). Many of the genes under the control of ABI3 were shown to be involved in protein and lipid storage, acquisition of seed DT, and recovery from desiccation in *Physcomitrella patens* (Khandelwal et al., 2010; Mönke et al., 2012). In *X. viscosa*, two genes have high homology with ABI3 and 32 genes

have high homology with members of the ABI3 regulon. Expression of most of them (81%) increases in shoots upon ABA treatment. It has been suggested that ABI3 is part of an evolutionary well-conserved regulatory network responsible for cellular drought tolerance (Mönke et al., 2012). Considering the essential role of ABI3 during seed maturation and acquisition of DT, it is likely that ABI3 has been recruited to regulate the desiccation processes required for the formation of a mature seed (Mönke et al., 2012). Therefore, the regulatory pathways coordinated by ABI3 might be fruitful targets for drought-improvement in crops.

Significant advances in improvement of stress tolerance in crops can also be made by sequencing new genomes. In **Chapter 6**, I present a draft genome of the resurrection plant *X. viscosa*, a monocotyledonous plant phylogenetically close to cereals. In the past decades, several studies of *X. viscosa* aimed at understanding its resurrection properties and utilizing them in the biotechnological production of more drought-tolerant cereal crops (Farrant et al., 2015). However, these studies have been hampered by the lack of an annotated genome sequence. Therefore, analysis of the genome sequence of *X. viscosa* may enable the transfer of knowledge of a new pool of abiotic stress target genes to the application of molecular-supported breeding efforts in cereals and other crops. For example, the *X. viscosa XVT8*, a stress-responsive dehydrin with chaperone activity, has already been identified and characterized (Ndima et al., 2001). Now, with the genome sequence available, its promoter sequence can be analysed to identify binding sites for transcription factors that may be crucial for its regulation during stress.

New genome sequences only provide useful information when the spatial and temporal expression, functions and interactions of gene products are well-characterised (Eldakak et al., 2013). Accordingly, transcriptome and proteome analysis of shoots and roots of *X. viscosa* seedlings before and after application of exogenous ABA allowed the identification of organ-specific changes in response to re-establishment of DT (**Chapter 6**).

Biotechnological approaches to improve drought stress in plants may involve overexpression of genes involved in particular aspects of DT, such as the ABA signalling pathway, osmotic adjustment or antioxidants. Although the overexpression of a single stress-responsive

gene can already contribute to drought resistance, ectopic expression or suppression of regulatory genes, together with drought-inducible or tissue-specific promoters, could be a more promising strategy due to the activation of multiple mechanisms of stress tolerance simultaneously (Deikman et al., 2012; Xiao et al., 2009).

Seeds and resurrection plants: two of a kind?

It has been suggested that pteridophytes represent a separate evolution of vegetative DT derived from a primitive form of DT and that angiosperms evolved as a modified form of DT from non-tolerant ancestors (Oliver and Bewley, 1997; Oliver et al., 2000). This hypothesis is based on the evolution in angiosperms of induced protection mechanisms capable of limiting water loss, invoking DT on demand (Oliver and Bewley, 1997; Oliver et al., 2000). Such a strategy would enable plants to direct resources away from growth or reproductive processes only when faced with desiccation at the cost of not surviving rapid dehydration (Oliver et al., 2000). On the other hand, fully desiccation tolerant plants were hypothesised to have evolved constitutive mechanisms that allow them to survive rapid dehydration instead of limiting it (Oliver et al., 2000). However, since this hypothesis was first postulated, new information became available, resulting in a different hypothesis.

It is very likely that the initial steps towards the colonisation by plants of land were taken by photosynthetic prokaryotes such as cyanobacteria, followed by fully aquatic eukaryotic algae adapted to live in muddy lake margins (Graham et al., 2012, 2014). These algae would be able to distinguish subaerial and aquatic conditions and adjust developmental processes and body morphology accordingly and reversibly (Graham et al., 2012). They would also be desiccation tolerant and the source of spore and body-DT (and other physiological traits useful in terrestrial habitats) of modern bryophytes (Graham et al., 2012, 2014). Then, during the evolution of vascular plants, the acquisition by sporophytes of a vascular system and a waxy cuticle with stomata meant the first steps towards drought resistance (Watkins et al., 2007).

Resisting drought allowed them to invest more time and energy in growth and reproduction, overcoming the slower growth speed characteristic of being desiccation tolerant. Nowadays, less than 1% of sporophytes of pteridophytes are desiccation tolerant (Pittermann et al., 2013). On the other hand, DT is widespread in gametophytes of pteridophytes. These gametophytes lack vascular tissues and have a poorly developed cuticle, resembling bryophytes (Watkins et al., 2007). For these gametophytes, being desiccation tolerant may have been critical in the radiation from protected terrestrial habitats into canopy and more drought-prone habitats, leading to increased longevity required for effective outcrossing (Watkins et al., 2007). Then, the evolution of a powerful regulation of stomata mediated by ABA in seed plants enhanced the survival of plants over both short- and long-term drought (Mcadam and Brodribb, 2013) and DT became definitely confined to pollen grains, spores and seeds. Later, during the evolution of angiosperms, the resurrection plants were able to bring DT back to vegetative tissues.

Therefore, I propose that the DT nowadays observed in pteridophytes and angiosperms evolved directly from bryophytes, adapting the mechanisms to a more complex body and physiology. In this sense, seed-DT would be orthologous to fern sporophyte DT. It is interesting to notice here that young sporophytes of the fern *Asplenium auritum* are desiccation tolerant and, during maturation, they lose DT, develop drought-avoiding mechanisms and a high capacity to grow in size (Testo and Watkins, 2012). A similar behaviour can be observed in orthodox seeds. The embryos inside orthodox seeds may be considered desiccation tolerant young sporophytes that lose DT already during germination. Moreover, these embryos/young sporophytes behave as resurrection plants, surviving cycles of hydration and dehydration as exemplified by Bai et al. (2012). In this sense, seeds and resurrection plants are two of a kind.

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Summary

The interest of research groups in desiccation tolerance (DT) has increased substantially over the last decades. The emergence of germinated orthodox seeds and resurrection plants as main research models has pushed the limits of our knowledge beyond boundaries. At the same time, new questions and new challenges were posed. The work presented in this thesis aims at shedding light on some of these questions, deepening our understanding of DT and providing relevant information to improve stress resistance in crops.

Chapter 2 is a survey of the literature and discusses the ecological and evolutionary significance for seeds to be able to re-acquire DT after germination. This chapter also discusses recent progress in DT studies using developing and germinated seeds of the model plants *Arabidopsis thaliana* and *Medicago truncatula*.

In **Chapter 3** I used microarray data from a time series of DT re-acquisition, together with network analysis of gene expression, to gain temporal resolution and identify relevant genes involved in the re-acquisition of DT in germinated *A. thaliana* seeds by incubation in abscisic acid (ABA). Overall, genes related to protection, response to stresses, seed development and seed dormancy were up-regulated, whereas genes related to cell growth and photosynthesis were down-regulated with time. Genes that respond early to exogenous ABA were related to wax biosynthetic processes, lipid storage, seed development and response to ABA stimulus. Genes that respond late to exogenous ABA were related to syncytium formation and response to abiotic stimulus (mainly light stimulus). The robustness of the network was confirmed by the projection of sets of genes – related to the acquisition of DT, seed dormancy, drought responses of adult plants and re-induction of DT by polyethylene glycol – on this network.

In **Chapter 4** the relation between DT in germinated seeds and drought resistance in adult plants is analysed, using rice (*Oryza sativa*) as experimental model. Considering the predictions of a future with lower availability of fresh water, efforts to increase rice drought tolerance without reducing yield are increasingly important. The results presented

in this chapter suggest that the intrinsic mechanisms of drought tolerance in adult plants are part of the mechanisms used by seeds to tolerate desiccation, but the molecular nature of these mechanisms remains elusive.

Chapter 5 explores the relation between DT and longevity in germinated seeds of the Brazilian tree species *Sesbania virgata* as experimental model. DT and longevity are acquired by orthodox seeds during the maturation phase of development and lost upon germination. DT can be re-induced in germinated seeds by an osmotic and/or ABA treatment, but there is no information on how these treatments affect seed longevity. *S. virgata* seeds lose DT slowly upon radicle growth. The radicle appeared to be the most sensitive organ and the cotyledons the most resistant. The ability to produce lateral roots was key for whole seedling survival. An osmotic treatment improved DT in germinated *S. virgata* seeds, but not longevity. This implies that DT and seed longevity can be uncoupled.

Xerophyta viscosa is one of the best studied resurrection species. Despite the fact that adult plants and mature seeds display DT, young *X. viscosa* seedlings are sensitive to fast drying. A treatment with ABA can induce DT early in shoots of these seedlings, but not in roots. **Chapter 6** addresses the changes in the transcriptome and proteome of *X. viscosa* seedlings during induction of DT. A draft genome sequence of *X. viscosa* was used to improve transcript and protein identification and annotation. Differences in ABA signalling and the cross talk between ABA and ethylene were presented as determinant for shoot and root responses. Moreover, differences in the accumulation of late embryogenesis abundant proteins were also shown as being key for DT in shoots and roots.

In **Chapter 7**, DT-transcriptomes of distantly related organisms are compared and surveyed for a core set of genes representing the signatures of critical adaptive DT mechanisms. A shortlist of 260 genes emerged, with a significant number of genes under the control of ABI3 and related to dormancy. The results reinforced the idea that core mechanisms and key regulators involved in DT developed early in the history of life and were carried forward by diverse species and life forms in a conserved manner and in conjunction with dormancy.

In **Chapter 8**, the findings of this thesis are integrated, showing

how they can contribute to future improvement of stress tolerance in crops. The ability of germinated seeds to re-acquire DT is discussed in combination with dormancy and longevity and related to seed survival under unfavourable environmental conditions. The relationship between drought- and desiccation tolerance and the role of ABA are presented briefly. Possible approaches to mine for new genes for crop improvement, such as searching for conserved genes and analysing new genome sequences, are addressed. Finally, a new perspective of the way to consider the evolution of DT is proposed.

Acknowledgements

The work presented in this thesis would not have been possible without the scientific and personal contribution and support of many people to whom I am deeply grateful. Opportunities to thank publicly all these people do not appear often.

Doing a PhD in the Laboratory of Plant Physiology (PPH) was a wonderful experience. I was lucky to be in an environment that promotes learning, creativity, friendship and well-being. Keeping such a nice environment with so many people from so many different cultural backgrounds is a big challenge and I am grateful to the efforts of all PPH staff members to promote it.

I especially thank Harro for encouraging diverse social activities in PPH and working hard to keep the science within high quality standards. I acknowledge your efforts to keep track of the progress of my research and I appreciate your critical reading and corrections on the thesis.

I would like to thank Richard, Dick and Sander for their advice and encouragement during my PhD.

Words cannot express my joy and pride to be part of the Wageningen Seed Lab. Feeling part of a group has never been so easy. I thank Henk, Wilco and Leónie for leading the group by example, creating an environment that makes us feel safe to express our ideas, challenges us to improve constantly and promotes union and friendship. I deeply admire you!

Working with Henk is a special honour. You kindly welcomed me in the Seed Lab and, since then, you have been a great mentor and friend. Thanks to your guidance, motivation and trust, I learned a lot and grew professionally. I hope to stay working with you for many more years, enjoying your enthusiasm and passion for science.

I own a big share of my achievements during my PhD also to Wilco. Your kindness, friendship and cheerfulness were a great comfort and inspiration to me. Your vast knowledge on pretty much everything pushed me to expand my own interests and develop more “T-shaped skills”. It was great to share with you many moments of scientific discussions as well as moments of entertainment and fun. I wish we keep sharing these

moments.

I hope more female junior scientists can be inspired by brilliant women the way I am inspired by Leónie, Jill Farrant and Julia Buitink. You stimulate me to pursue the dream of a scientific career, knowing that it is possible to achieve it. I deeply admire you.

I would like to thank Rina, Margaret and the EPS staff members for always being helpful and fast in dealing with all the administrative-related issues.

I would like to thank the students that worked with me. I really appreciate your contribution to my work and my development.

Life abroad can be very hard. If you are doing a PhD, it can be even harder. I was lucky to be surrounded by great people who made my life in Wageningen so pleasant. I will always carry with me good memories of all of you. Thank you for the uncompromised chats over lunch and coffee, for the relaxing time enjoying the Dutch summer, for the late-afternoon venting. Thank you for the free therapy sessions. Thank you for all the help at the lab and the scientific chats. Thank you for being my gym buddies. Thank you for being great neighbours. Thank you for the multicultural environment. Thank you for all the fun, the music and the food. Thank you for all the hugs. Thank you for the Beer sessions, Bowling evenings, Lab Trips, Spanish and Dutch Lunches, WeDays, Crochet sessions, volleyball games, golfing, workshops, concerts, movies, dinners, parties, etc.

I was especially fortunate to have met my dear friends and paranimphs Natalia and Phuong. Your friendship means a lot to me. You are very strong women with a great future ahead. Thanks for always being there for me, for your patience, kindness and wisdom. I will always treasure every moment we spent together. You are awesome!

A parte mais difícil de morar em outro país é a distância daqueles que mais amamos. Gostaria de agradecer aos amigos brasileiros que fiz em Wageningen por aliviarem as saudades enormes de casa. A convivência com vocês significa muito para mim. Gostaria também de agradecer aos amigos que deixei no Brasil, que nunca deixaram de estar presentes e me apoiar. Conto os dias para ver vocês de novo!

Eu tenho a imensa sorte de ter nascido em uma família maravilhosa. Agradeço o amor incondicional dos meus pais. Minhas escolhas podem

ter me levado por um caminho diferente do que vocês imaginaram para mim, mas vocês me preparam para construir minha felicidade independentemente de qualquer decisão. Agradeço meus irmãos pela amizade e carinho. Agradeço meus avós pelas conversas ao telefone e o amor. Agradeço especialmente minha avó Véra por ter aceito o desafio de desenhar a capa da minha tese. Eu tinha certeza de que a Senhora faria um excelente trabalho! Agradeço minha madrinha Dylia pela atenção e preciosa amizade. Agradeço toda a família pelo apoio e suporte. Mal posso esperar pelo nosso reencontro!

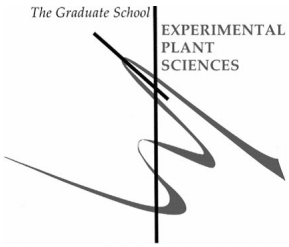
Finalmente, o trabalho apresentado nessa tese não teria sido possível sem o auxílio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasil).

About the author

Maria Cecília Dias Costa was born on 16 September, 1984 in Belo Horizonte, Brazil. In 2004, she joined the undergraduate program in Biology at the Federal University of Minas Gerais, Brazil. During her studies, she did internships in different fields of biology, such as environmental education, wastewater treatment, and plant science. Her first experience in the field of plant science was in the project "Structural and physiological impacts of insect galls in leaves of *Aspidosperma australe* and *Aspidosperma spruceanum*", under the supervision of Dr José Pires de Lemos Filho. Subsequently, she worked at the botanical garden of the Belo Horizonte Zoo-Botanical Foundation, where she had the opportunity to deepen her knowledge on Brazilian native plant species. During this period, she had her first experience with seed biology, working with seeds of Brazilian native species of *Anthurium*, under the supervision of Dr Queila de Souza Garcia. These seeds seemed to be desiccation sensitive. Further investigations about them were interrupted by an internship at the Autonomous University of Barcelona, Spain, when Maria Cecília also did volunteer work at the Botanical Garden of Barcelona. Back to Brazil, the experience with desiccation sensitive seeds led her to start an MSc at the Federal University of Lavras, Brazil. During her MSc, she worked with desiccation tolerance and storage of germinated seeds, under the supervision of Dr José Marcio Rocha Faria. Due to her increasing interest on desiccation tolerance, Dr José Marcio encouraged her to pursue a PhD degree at the Wageningen Seed Lab, The Netherlands. In 2012, under the supervision of Dr Henk Hilhorst and Dr Wilco Ligterink, she started working on the project that resulted in this PhD thesis.

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to:	Maria Cecília Dias Costa
Date:	8 January 2016
Group:	Laboratory of Plant Physiology
University:	Wageningen University & Research Centre

1) Start-up phase	**date**
► **First presentation of your project**	
Conserved regulation of desiccation tolerance in seeds and resurrection plants	Oct 10, 2012
► **Writing or rewriting a project proposal**	
Conserved regulation of desiccation tolerance in seeds and resurrection plants	Feb, 2012
► **Writing a review or book chapter**	
Acquisition and loss of desiccation tolerance in seeds: from experimental model to biological relevance, Planta, 241: 563-577	Oct, 2014
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	**date**
► **EPS PhD Student Days**	
EPS PhD student day, University of Amsterdam	Nov 30, 2012
EPS PhD student day, Leiden University	Nov 29, 2013
► **EPS Theme Symposia**	
EPS theme 1 'Developmental Biology of Plants'	Jan 19, 2012
EPS theme 3 'Metabolism and Adaptation'	Apr 26, 2012
EPS theme 3 'Metabolism and Adaptation'	Mar 22, 2013
EPS theme 3 'Metabolism and Adaptation'	Mar 11, 2014
EPS theme 4 'Genome Biology'	Dec 03,2014
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plan Sciences', Lunteren (NL)	Apr 02-03, 2012
ALW meeting 'Experimental Plan Sciences', Lunteren (NL)	Apr 22-23, 2013
ALW meeting 'Experimental Plan Sciences', Lunteren (NL)	Apr 13-14, 2015

►	**Seminars (series), workshops and symposia**	
	EPS Flying Seminar x7	2012-2015
	Master Class on Seed Technology	May 21-24, 2012
	ExPectationS Day 2013	Feb 01, 2013
	2nd Dutch Seed Symposium	Oct 01, 2013
	New Frontiers in Anhydrobiosis, Pornichet, France	Mar 23-27, 2014
	ExPectationS Day 2014	Mar 28, 2014
	WGS PhD Carousel Workshop	Jun 02, 2014
	3rd Dutch Seed Symposium	Aug 07, 2014
	Omics Advances for Academia & Industry	Dec 11, 2014
	From Big Data to Biological Solutions	Jun 18, 2015
►	**Seminar plus**	
	Prof. Dr. Jill Farrant	Jun 25, 2012
	Dr. Ruth Finkelstein	Nov 14, 2012
	Prof. Dr. Yves van de Peer	Feb 03, 2015
►	**International symposia and congresses**	
	4th Workshop on the Molecular Aspects of Seed Dormancy and Germination, Paris, France	Jul 09-12, 2013
	11th Conference of the ISSS, Changsha, China	Sep 15-19, 2014
	Plant Genome Evolution, Amsterdam (NL)	Sep 06-08, 2015
►	**Presentations**	
	Poster presentation at ALW meeting	Apr 22-23, 2013
	Plant Sciences SLAM at ALW meeting	Apr 22-23, 2013
	Oral presentation at "New Frontiers in Anhydrobiosis", Pornichet	Mar 23-27, 2014
	Oral presentation at "11th Conference of the ISSS", Changsha	Sep 15-19, 2014
	Oral presentation at EPS Theme 4 Symposium, Wageningen	Dec 03, 2014
	Oral presentation at "96th Dies Natalis, Wageningen	Mar 10, 2014
	Poster presentation at ALW meeting	Apr 13-14, 2015
	Oral presentation at PhD Trip	Apr 22-May 01, 2015
	Poster presentation at "Plant Genome Evolution", Amsterdam	Sep 06-08, 2015
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Sep 29, 2014
►	**Excursions**	
	PPH PhD trip	April 22-May 1, 2015
	Subtotal Scientific Exposure	*24.8 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
The power of RNA-Seq	Jun 05-07, 2013
Bioinformatics - A user's approach	Aug 27-31, 2012
System biology: Statistical Analysis of ~omics data	Dec 10-14, 2012
Transcription factors and transcription regulation	Dec 17-19, 2013
Data Carpentry Workshop	Jun 24-25, 2015
► **Journal club**	
PPH Literaure Discussion	2012-2015
► **Individual research training**	
INRA-Angers (France)	Nov 11-15, 2013
Subtotal In-Depth Studies	*10.0 credits**

4) Personal development	date
► **Skill training courses**	
Techniques for writing and presenting a scientific paper	Apr 01-04, 2014
Competence Assessment	May - Jun 19, 2012
Voice Matters - Voice and Presentation Skills Training	Apr 09 & 23, 2013
Project and Time Management	May - Jun , 2013
Guide to Scientific artwork	Apr 14-15, 2014
Writting Grant Proposals	Apr - May - Jun 2015
Reviewing a scientific paper	Sep 17, 2015
► **Organisation of PhD students day, course or conference**	
Organization of the PPH PhD trip	Apr 22-May 02, 2015
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*7.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**55.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was financially supported by the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq, Brazil).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover by Vera Lisardo Dias (digital adaptation of oil pastel)

Thesis layout by Maria Cecília D. Costa and Thijs Dueck

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