INTEGR. COMP. BIOL., 45:788–799 (2005)

Desiccation Tolerance in Bryophytes: A Reflection of the Primitive Strategy for Plant Survival in Dehydrating Habitats?[1]

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SYNOPSIS. Bryophytes are a non-monophyletic group of three major lineages (liverworts, hornworts, and mosses) that descend from the earliest branching events in the phylogeny of land plants. We postulate that desiccation tolerance is a primitive trait, thus mechanisms by which the first land plants achieved tolerance may be reflected in how extant desiccation-tolerant bryophytes survive drying. Evidence is consistent with extant bryophytes employing a tolerance strategy of constitutive cellular protection coupled with induction of a recovery/repair mechanism upon rehydration. Cellular structures appear intact in the desiccated state but are disrupted by rapid uptake of water upon rehydration, but cellular integrity is rapidly regained. The photosynthetic machinery appears to be protected such that photosynthetic activity recovers quickly. Gene expression responds following rehydration and not during drying. Gene expression is translationally controlled and results in the synthesis of a number of proteins, collectively called rehydrins. Some prominent rehydrins are similar to Late Embryogenesis Abundant (LEA) proteins, classically ascribed a protection function during desiccation. The role of LEA proteins in a rehydrating system is unknown but data indicates a function in stabilization and reconstitution of membranes. Phylogenetic studies using a *Tortula ruralis* LEA-like rehydrin led to a re-examination of the evolution of desiccation tolerance. A new phylogenetic analysis suggests that: (i) the basic mechanisms of tolerance seen in modern day bryophytes have changed little from the earliest manifestations of desiccation tolerance in land plants, and (ii) vegetative desiccation tolerance in the early land plants may have evolved from a mechanism present first in spores.

INTRODUCTION

Green plants are believed to have colonized the land from a fresh water origin (Mishler and Churchill, 1985), requiring adaptive mechanisms that permit avoidance and/or survival of dehydration. In the initial ventures into the dehydrating atmospheres of land habitats, plants were of a very simple architecture and had yet to evolve more complex morphological or physiological strategies to prevent water loss. Water would have been quickly lost from the cells of these plants once it was no longer present in liquid form around them. Thus primitive land plants would, in all likelihood, spend a significant amount of time in equilibrium with the surrounding air, which in most cases would mean that the plants would be desiccated. Even at a relative humidity of 50% (at 28°C) a plant when equilibrated would experience a water potential of approximately −100 MPa (Gaff, 1997), a water deficit that is lethal to the majority of modern day flowering plants. Early land plants would have had to evolve mechanisms to survive such harsh drying treatments in order to have successfully exploited habitats on land. Simply put, we hypothesize that primitive plants must have been desiccation-tolerant in both vegetative and reproductive stages in order to colonize the land.

If this is so, what can we learn about this earliest of adaptive traits that shaped the evolution of land plants? How has the ability of plants to survive desiccation evolved over time? These are not simply esoteric questions. The answers we might discover not only have evolutionary importance, they have practical application as well. The ability of plant cells to respond to and cope with severe water deficits has economic and agricultural implications that directly relate to crop productivity in an ever challenging and changing environment. An understanding of these responses and tolerance mechanisms could be vital if we are to respond to the increasing need for a stable and sufficient food supply.

Vegetative desiccation tolerance is broadly distributed among modern day plant taxa (Alpert, 2000). Tolerance is relatively common, but not universal, in the bryophytes (Proctor, 1990; Proctor and Pence, 2002), but it is much rarer in vascular plants (tracheophytes), plants that developed the morphological adaptations required to transport water, *i.e.*, tracheids and vessel elements. Porembski and Barthlott (2000) estimate that there are only about 300 desiccation-tolerant species of tracheophytes. As discussed in the final section of this paper, phylogenetic analyses suggest that with the evolution of the tracheophytes, vegetative desiccation tolerance was lost (Oliver *et al.*, 2000, see below) and that its occurrence in a few clades of tracheophytes represents independent evolutions (or re-evolutions), presumably in response to selection pressures associ-

[1] From the Symposium *Drying Without Dying: The Comparative Mechanisms and Evolution of Desiccation Tolerance in Animals, Microbes, and Plants* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 4–8 January 2005, at San Diego, California.

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ated with arid niches. By investigating the underlying mechanisms that allow these plants to survive the extreme water deficits that characterize desiccation, we can gain an understanding of how this trait has evolved and what processes are important in its establishment or acquisition. In keeping with the view that plants and plant tissues achieve desiccation tolerance by virtue of the inherent properties of their cellular components (protoplasm), as discussed by Bewley (1979), this discourse will center on the cellular mechanisms that characterize the various forms of vegetative tolerance in land plants. Furthermore, the intention is to focus on the mechanisms of tolerance exhibited by bryophytes and to compare them to the tolerance mechanisms of angiosperms to infer the possible adaptive nature of the various aspects of the response of these plants to desiccation. It is in the study of bryophytes that we can come closest to learning how early plants established themselves in the potentially lethal dehydrating terrestrial habitats as they colonized the land. Comparisons with the mechanisms for tolerance that have evolved in the morphologically more complex tracheophytes allow for the generation of hypotheses concerning the evolution and ecology of this important trait and underlying genetic and physiological processes.

MECHANISTIC CONSIDERATIONS

Bewley (1979) outlined three protoplasmic properties of plant cells that have to be present for desiccation tolerance to be established: 1. Limit damage from desiccation and/or rehydration to a minimum, 2. Maintain cellular integrity in the desiccated state, and 3. Activate or mobilize repair mechanisms upon rehydration. Basically these criteria translate into tolerance mechanisms that protect and/or repair plant cells such that desiccation or rehydration damage is ultimately negated. Several possible strategies for desiccation tolerance can be proposed based on the two basic processes of cellular protection and repair. Plant cells can either constantly maintain the processes and components necessary to protect cells, rendering desiccation tolerance a constitutive trait, or these can be induced when dehydrating conditions are encountered. Similarly the processes and activities associated with repair could be constitutive or induced following rehydration. As both cellular protection and repair are likely to be essential for any mechanism of desiccation tolerance, all combinations of constitutive *versus* inducible for these two elements are possible. In addition, as discussed above, since vegetative desiccation tolerance within land plants has evolved (or re-evolved) multiple times it is theoretically possible that we could encounter any one of the possible variations mentioned above when investigating the individual mechanisms exhibited in different species of tolerant plants. However, it seems reasonable to assume that since tolerance mechanisms have evolved from a shared ancestral strategy, later manifestations of this trait have evolved mechanisms that are fundamentally similar. As will be discussed later, from the evidence available to us, this does appear to be the case.

The actual components and processes involved in both cellular protection and repair are in all probability highly conserved (at least in function), constrained by the underlying physics associated with the desiccation of plant cells. Indeed, from what we do know this appears to be true, at least for the components involved in cellular protection (see below and Oliver *et al.*, 2000; Phillips *et al.*, 2002; Buitink *et al.*, 2002). However, much needs to be done in this area before we can fully understand either of these processes.

Desiccation tolerance in bryophytes

Bryophytes today experience an environment, at least with regards to water relations, that we postulate ancient plants faced when the land was first colonized (see above). Water transport occurs externally to the plant, which is generally one cell layer thick and water is freely lost to or gained from the surrounding habitat across the cell membrane. This rapid and direct equilibration of cell water content to that of the environment is called *poikilohydry*. When free water is depleted from the surface of the plant the leaf cells immediately moves towards equilibrium with the water potential of the surrounding air; the plant desiccates. Depending upon the relative humidity of the air this can be a slow or rapid progression to equilibrium and dryness; the higher the humidity the slower the drying rate. The extent of water loss also depends upon the relative water content of the air and its temperature (see the example given earlier). As discussed in detail by Proctor and Pence (2002), most bryophytes can survive moderate levels of desiccation (to −20 to −40 MPa) for short periods, certainly beyond the range that most crop species can survive (−1.5 to −3 MPa). Some bryophytes however, can tolerate severe desiccation and for extended periods, for example *Tortula caninervis*, a desert species, can remain at around −540 MPa (equilibrated to the atmosphere above activated silica gel; 2–4% RH) for up to six years and still recover normal activity and growth (Oliver *et al.*, 1993; unpublished data). The drying rate, length of desiccation, intensity of desiccation, prior dehydration (hardening), and temperature, all have an effect on the ability of desiccation-tolerant bryophytes to recover from the drying event (extensively reviewed by Proctor and Pence, 2002). Drying rates can be very rapid in exposed habitats but clump architecture can slow water loss such that in general mosses can exert a rudimentary control over the drying rates (Proctor, 1980; Rice *et al.*, 2001). In the Organ Mountains of New Mexico, clumps of *Tortula ruralis* would sustain hydration for 3 to 4 hr when artificially moistened and subsequently reach an equilibrated dryness at 6 hr (unpublished observations). Experimentally, many bryophytes can survive extremely rapid desiccation, *i.e.*, to −540 MPa in less than 30 min. Rehydration is almost instantaneous (30 to 90 sec) and recovery rates are also generally very rapid with most bryophytes reaching

full recovery within a few hours. The recovery of some processes, such as photosynthesis and respiration, can be extremely rapid: in the case of photosystem II, recovery occurs within a few minutes (Proctor, 2001). The rate of recovery does, however, depend upon the rate at which the prior desiccation occurred (Oliver and Bewley, 1997). In contrast, all desiccation-tolerant angiosperms only survive such drying events if the rate of water loss is very slow (in days to weeks) and take up to 24 hr or more to recover (Oliver and Bewley, 1997; Alpert and Oliver, 2002; Proctor and Pence, 2002).

With their strategic phylogenetic positioning, descended from early divergence events in the history of land plants, bryophytes are ideally suited as models for understanding how primitive plants survived the rigors of colonizing dehydrating and else wise stressful habitats as they moved onto the land. That bryophytes can endure such extremes of dehydration and recover so quickly is a testament to the effectiveness of the cellular strategy for desiccation tolerance these plants evolved.

Bryophyte Mechanism for Desiccation Tolerance

Many desiccation-tolerant bryophytes do apparently share a common mechanism for the tolerance of cellular dehydration. Of the desiccation-tolerant bryophytes that have been extensively studied, mainly the highly tolerant species of the genus *Tortula* (in particular *T. ruralis*), all appear to utilize a mechanism that directs the constitutive protection of cellular structures coupled with a rehydration-induced repair/recovery process (Oliver and Bewley, 1997; Oliver *et al.*, 2000; Alpert and Oliver, 2002). The evidence for this conclusion has been recently and extensively reviewed in the literature (Oliver *et al.*, 2000; Alpert and Oliver, 2002). In this report we will summarize this evidence along with the addition of some new data that is directed at both an understanding of the tolerance mechanism exhibited by bryophytes and its place in the evolution of this trait among land plants.

Constitutive Cellular Protection

The presence of a constitutive cellular protection component to the bryophyte mechanism for desiccation tolerance can be inferred by their ability to tolerate rapid desiccation events. Desiccation, and the ensuing metabolic quiescence, can occur too quickly to initiate and establish cellular protective measures. Protein synthesis, a prerequisite for most stress response mechanisms in plants, is extremely sensitive to cellular dehydration and is rapidly lost during drying of *T. ruralis* gametophytes (reviewed by Bewley, 1979; Bewley and Krochko, 1982). Oliver (1991) clearly demonstrates that there are no novel transcripts recruited by the protein synthetic machinery during drying. In contrast, desiccation-tolerant angiosperms cannot survive desiccation if water loss occurs rapidly (less than 12 hr) and it is well established that the mechanism of tolerance in these plants utilizes a dehydration-induced cellular protection strategy requiring both novel transcription patterns and new protein synthesis (see below, Ingram and Bartels, 1996; Oliver *et al.*, 2000; Alpert and Oliver, 2002; Phillips *et al.*, 2002). Apart from the metabolic considerations, ultrastructural observations strongly suggest that membranes of vegetative cells of desiccation-tolerant bryophytes do not suffer observable damage during drying. Freeze-fracture electron microscopy has enabled the ultrastructural investigation of dried plant cells to progress with little fear of artifact generation and was used successfully to demonstrate that membranes in seeds and pollen retain normal lipid bilayer organization at very low water contents (Thompson and Platt-Aloia, 1982; Platt-Aloia *et al.*, 1986). Similar studies with *T. ruralis*, and the spike moss *Selaginella lepidophylla*, demonstrated that this is also the case in the cells of the leaf tissues of these two plants (Platt *et al.*, 1994). Finally there is also biochemical evidence to support a constitutive cellular protection strategy in bryophytes. In orthodox seeds and vegetative tissues of desiccation-tolerant angiosperms two cellular components, the Late Embryogenesis Abundant (LEA) proteins and soluble sugars accumulate in response to desiccation (for review see Phillips *et al.*, 2002; Buitink *et al.*, 2002; Kermode and Finch-Savage, 2002). These two components are generally considered critical in the acquisition of cellular desiccation tolerance, although the actual function of the LEA proteins remains unclear (Cuming, 1999). The genes encoding the Group II LEA proteins, termed dehydrins, are generally induced in response to water deficits (Close, 1997) and in the case of *Craterostigma plantagineum*, a desiccation-tolerant angiosperm, during desiccation (Ingrams and Bartels, 1996) resulting in the accumulation of dehydrin proteins in the drying cells. In *T. ruralis* dehydrins are apparently constitutively expressed, at least at the protein level (Bewley *et al.*, 1993). The accumulation of soluble sugars has long been correlated with the acquisition of desiccation tolerance in plants and other organisms (Crowe *et al.*, 1992; Vertucci and Farrant, 1995). In seeds, pollen, and most plants that accumulate soluble sugars in response to desiccation utilize the disaccharide, sucrose. In *Craterostigma plantagineum*, 2-octulose stored in the hydrated leaves is converted to sucrose during drying to such an extent that in the dried state it comprises about 40% of the dry weight (Bianchi *et al.*, 1991). Sugars are a major contributing factor to vitrification (biological glass formation) of the cytoplasm of dried cells (Buitink *et al.*, 2002). Sucrose makes up approximately 10% of the dry mass of *T. ruralis* gametophytes and does not change in amount during desiccation or rehydration in the dark or light (Bewley *et al.*, 1978). Thus, it appears important to maintain a constant, and presumably sufficient, amount of this sugar in this moss. The lack of an increase in soluble sugars in response to desiccation appears to be a common feature of tolerant mosses (Smirnoff, 1992).

CELLULAR DAMAGE: DESICCATION OR REHYDRATION?

If vegetative bryophyte cells are essentially intact in the dried state as the evidence seems to indicate, what damage is there that would require a rehydration-induced (or activated) repair system as first suggested by Bewley (1979)? Cellular damage could occur during desiccation, as has been seen in sensitive tissues (Walters *et al.*, 2002), which are associated with such activities as lipid oxidation and free radical generation. In the bryophyte studies we know that such activities occur (Smirnoff, 1992) but, as is obvious from the ultrastructural observations, visible and extensive membrane damage is not evident. We do know that membranes are altered in desiccation-tolerant tissues during desiccation; *e.g.*, Buitink *et al.* (2000), using electron paramagnetic resonance (EPR) spectroscopy, demonstrated that tolerant tissues differ from sensitive tissues in the partitioning of amphiphilic substances into membranes. Cellular damage can also occur during extended periods in the dried state through oxidative and other chemical activities, the extent of damage depends on the degree of desiccation and time (Walters *et al.*, 2002; Buitink *et al.*, 2002). Cellular damage also occurs during the inrush of water as rehydration progresses. Since any damage caused by the desiccation phase is only manifest following rehydration, it is difficult to say which of the two processes of dehydration or rehydration is the root cause of the damage. Rehydration is certainly damaging to dried cells and can result in significant injury that desiccation-tolerant systems strive to prevent (Osborne *et al.*, 2002). Orthodox seeds are capable of slowing the rehydration process, allowing for some order in an otherwise chaotic event. Bryophytes however, have no such capability and rehydrate almost instantaneously when water is added. The first indication that there is an alteration in the cellular structure during rehydration of dried plant cells is the leakage of solutes from the protoplasm. In desiccation-tolerant tissues this leakage is transient and the extent of leakage is dependent upon the rate at which the prior desiccation event occurred. The faster the drying rate the more solutes are leaked during rehydration (Bewley and Krochko, 1982; Oliver and Bewley, 1984; Oliver *et al.*, 1993). It is generally accepted that membrane phase transitions are the cause of rehydration leakage in vegetative tolerant tissues (Crowe *et al.*, 1992).

As water enters the dried cells of *T. ruralis* the condensed cytoplasm rapidly expands to fill the empty cell cavity formed by plasmolysis (Tucker *et al.*, 1975) Within five minutes chloroplasts are swollen and globular in shape and their outer membranes are folded and separated from the thylakoids, which themselves are no longer compacted (Tucker *et al.*, 1975; Bewley and Pacey, 1978). The extent of thylakoid disruption is dependent upon the prior speed of desiccation; the more rapid the drying rate the more disruption occurs. Mitochondria also swell and exhibit disruption of the internal membrane structures (cristae), but the appearance of this organelle upon rehydration is not affected by the rate of desiccation. Similar results have been reported for other desiccation-tolerant moss species (reviewed by Oliver and Bewley, 1984). It has been suggested that such alterations in cellular structure observed following rehydration as simply artifacts of fixation procedures used on rehydrating dried materials, especially as many of these studies were reported in the early 1980s. In the case of the ultrastructural studies concerning *Tortula* this seems unlikely as fixation was delayed until 5 minutes following the re-addition of water so that cells were fully hydrated, and similarly treated hydrated control tissues were apparently unaffected by the methods of fixation used in these studies. In all cases organelles regain normal structure within 24 hr of the readdition of water. Rehydrated cells of dried gametophytes of the desiccation-sensitive moss *Cratoneuron filicinum* exhibit identical structural abnormalities as those seen in *T. ruralis* but in this case the cells never regain a normal appearance and die (Bewley and Pacey, 1978; Krochko *et al.*, 1978). Desiccation and rehydration (imbibition) induced damage has been extensively investigated in desiccation-tolerant seeds and pollen (reviewed by Osborne *et al.*, 2002; Walters *et al.*, 2002) and indicates not only damage and alterations in membranes but also cytoskeleton, nucleus, and DNA (chromatin). In bryophytes we have little direct evidence for cellular damage other that what has been observed ultrastructurally.

INDUCED OR ACTIVATED CELLULAR REPAIR

Much of what we can determine about the repair or reconstitution requirement in bryophytes has been inferred from physiological, biochemical, and genetic responses that have been monitored during the initial phases of recovery following rehydration. As eloquently stated by Proctor and Pence (2002) ''full recovery must involve a diversity of processes'' and as is becoming clear, the complexity of recovery and repair will be difficult to detail and characterize.

Early work (see Bewley, 1979 for review) established the ability of *Tortula ruralis*, a primary model for these studies, and other mosses to rapidly recover synthetic metabolism when rehydrated. This rapid recovery is in part possible because of the efficient sequestration of the components of protein synthesis, with the exception of some initiation factors, during drying (Oliver and Bewley, 1997). During the first two hours following rehydration of dried *T. ruralis* there is an extensive alteration in gene expression as measured by changes in the patterns of proteins synthesized during this time compared to hydrated controls. By 2D gel analysis Oliver (1991) was able to detect the termination or decrease in synthesis of 25 proteins (termed hydrins) and the initiation or substantial increase in the synthesis of 74 others (termed rehydrins). Although this is, of course, an underestimate of the rehydration-manifested alterations in protein synthesis this study was able to demonstrate that the controls over changes in synthesis of these two groups of pro-

teins are not mechanistically linked. It takes a certain amount of prior water loss to fully activate the synthesis of rehydrins upon rehydration. Perhaps this is a strategy that has evolved to link the amount of energy expended in repair to the amount of damage potentiated by differing extents of drying.

Oliver (1991) also demonstrated that the alterations in the protein patterns synthesized following rehydration of *Tortula* gametophytes occur within a background of a qualitatively unaltered transcript population indicating that the change in gene expression with rehydration is brought about by a change in translational controls. Using cDNA clones corresponding to *T. ruralis* rehydrins, Scott and Oliver, (1994) confirmed that the basis of the control of gene expression centered upon translation and not transcription, and this has since been further validated in genomic level studies (see below). This is in direct contrast to the case in desiccation-tolerant angiosperms that utilize a desiccation induced change in transcriptional control to direct the synthesis of genes required for desiccation tolerance (Ingram and Bartels, 1996; Phillips *et al.*, 2002). However, the manner in which the control of translation is achieved in *Tortula* does appear to depend upon the previous rate of desiccation. Rehydrin transcripts accumulate for the first hour following rehydration of rapid-dried gametophytes, apparently to replenish a transcript pool that has been reduced by rapid desiccation (Scott and Oliver, 1994; Wood and Oliver, 1999; Velten and Oliver, 2001). In contrast, rehydrin transcripts accumulate during the drying phase if desiccation is slow, *i.e.,* 4 to 6 hr (Oliver and Wood, 1997; Wood and Oliver, 1999). Further studies clearly demonstrate that these transcripts accumulate not as a result of transcription but because they are selectively sequestered in mRNP particles (Wood and Oliver, 1999). Upon rehydration it is postulated that these transcripts are rapidly released and utilized for the rapid synthesis of rehydrins. This is supported by their rapid inclusion in the polysomal fraction of rehydrated gametophytes (Scott and Oliver, 1994; Wood and Oliver, 1999). These findings suggest that the repair mechanism in rehydrated slow-dried gametophytes, perhaps reflecting the natural response, is activated rather than induced (which implies a de novo assembly of components). The repair mechanism in rehydrated rapid-dried gametophytes would have to rely upon what little has been assembled until new components can be synthesized and assembled. This would be consistent with the slower recovery time and more visible damage associated with rehydrated rapid-dried gametophytes. There is a caveat to this postulate in that because rapid desiccation is achieved in very dry atmospheres it may also reflect the depth of desiccation achieved in rapid-dried gametophytes.

A number of recent physiological studies of the recovery of photosynthesis following rehydration of a number of desiccation-tolerant mosses have questioned the need for protein synthesis and repair with respect to the recovery of chloroplasts and chloroplast functions (reviewed by Proctor and Pence, 2002; Proctor and Tuba, 2002). Essentially, recovery of chloroplastic function, as measured by chlorophyll fluorescence measurement (Fv/Fm), is extremely rapid (10 to 20 min) and is unaffected by protein synthesis inhibitors in the dark for rehydrated dried gametophytes (dried to −70 MPa) of several desiccation-tolerant mosses (Proctor and Smirnoff, 2001; Proctor, personal communication). Protein synthesis is required however if rehydration occurs in the light, presumably to repair photo-oxidative damage. CO_2 uptake and assimilation, however, is not instantaneous and does require protein synthesis to recover. Measurements of the rapid recovery of photosynthesis upon rehydration for dried *T. ruralis* also suggest that protein synthesis may not be required for the recovery of chloroplast structure and function (Tuba *et al.*, 1996; Csintalin *et al.*, 1999). There are some caveats to these studies. The effectiveness of protein synthesis inhibitors is difficult to assess as they can also affect the rate of uptake of the radiolabelled amino acids used to assess protein synthetic rates. Protein synthesis inhibitors also rarely completely prevent protein synthesis even when used in combination. Proctor and Smirnoff (2001) report an effective inhibition of 90% after 20 min in the presence of two inhibitors (to inhibit both cytoplasmic and organellar synthesis), however, no uptake measurements or amino acid pool size measurements were reported. The level of desiccation obtained in the mosses used in these investigations is generally relatively moderate, approximately −70 MPa and as discussed above the rate and depth of desiccation has a major effect on the amount of cellular damage seen in bryophytes. Even with these considerations it does appear that the photosynthetic machinery (at least Photosystem II) is well protected in the dried state and may require little repair. It is interesting to speculate that, because of the need to rapidly utilize the time that water is available and the need for energy to repair other cellular damage, part of the bryophyte mechanism of tolerance lies in a focused and effective protection of the chloroplast.

Before progressing into some of the latest genomic-level studies it is worth mentioning that desiccation tolerance can be induced in some bryophytes, those that are not constitutively tolerant and live in mesic habitats. Beckett (1999) demonstrated that desiccation tolerance (as measured by ion leakage) of a mesic *Atrichum* species could be increased by a previous drying treatment and that addition of abscisic acid (ABA) to the hydrated moss could produce the same increase in tolerance. Several studies have demonstrated that ABA treatment of *Funaria hygrometrica* not only increases tolerance to desiccation, allowing for survival of rapid desiccation that is normally lethal, but also results in the induction of the synthesis of a number of proteins that accumulate during drying (Werner *et al.*, 1991; Bopp and Werner, 1993). ABA has similar effects on the tolerance of some liverworts to desiccation (Hellwege *et al.*, 1994) and its precursor lunularic acid con-

trols the switch from a sensitive to a tolerant stage of *Lunularia cruciata* (Schwabe and Nachomony-Bascomb, 1963).

The involvement of ABA and tolerance in these bryophytes is of interest because it is reminiscent of the strategy used by desiccation-tolerant angiosperms that involves, at least in part, the induction of gene expression associated with the acquisition of desiccation tolerance by an elevation in endogenous ABA (reviewed by Phillips *et al.*, 2002). It is ABA that is thought to be a major hormonal component to the induction of the cellular protection system during drying in seeds and most, if not all, desiccation-tolerant tracheophytes (Oliver *et al.*, 2000). Interestingly ABA was undetectable in *T. ruralis* gametophytes in either the hydrated or drying stages of the wet-dry-wet cycle and was not detected in dried tissues and ABA does not induce dehydrin accumulation in hydrated gametophytes (Bewley *et al.*, 1993; Oliver, unpublished observations). This again illustrates the complexity of the desiccation tolerance phenotype, and raises some interesting evolutionary questions such as: Did mesic bryophytes forego the constitutive cellular protection aspect of desiccation tolerance in favor of an inducible system that allows them to better compete in a mesic habitat? Or, did the constitutive cellular protection system evolve from a primitive developmental system in spores that allowed some mosses to move into progressively more extreme xeric habitats? Did the developmentally programmed acquisition of desiccation tolerance in seeds, that apparently utilizes an ABA induction pathway, evolve from an environmentally induced mechanism similar to that seen in mesic bryophytes? Only a greater understanding of the underlying genetic aspects of this interesting trait will enable us to answer such questions as these.

GENOMIC APPROACH TO THE REPAIR ASPECTS OF DESICCATION TOLERANCE IN BRYOPHYTES

One approach to understanding the full nature of the desiccation-rehydration response in bryophytes is to determine what transcripts are available during the wet-dry-wet continuum and which are targeted for translation. As we have discussed, in desiccation-tolerant bryophytes the gene expression response to desiccation and rehydration is controlled at the level of translation. To study this requires a genomics level approach combining EST based assessments of transcript populations coupled with microarray profiling assays to determine not only the available amount of any particular transcript but also its recruitment potential for protein synthesis.

It is only recently that bryophytes have garnered attention in regards to genomics in any form, and much of this has centered on the development of *Physcomitrella patens*, a desiccation-sensitive species, as a plant model for development and genetics (Kamisugi and Cuming, 2004; Holtorf *et al.*, 2004; Fujita *et al.*, 2004). For desiccation-tolerant species only *Tortula ruralis* has served as a model for genomic level investigations (Wood and Oliver, 2004). In small-scale studies we isolated 18 rehydrin cDNAs (Scott and Oliver, 1994) followed by a further 152 ESTs from a cDNA expression library made from sequestered mRNAs extracted from slow-dried gametophytes (Wood *et al.*, 1999). Only 29% of these cDNAs demonstrated any significant similarity to previously identified nucleotide and/or peptide sequences. Of those that could be assigned a possible identity and putative function several were ribosomal proteins (perhaps indicative of the role of translation in the response), early-light inducible proteins (ELIPs), and desiccation-related polypeptides, in particular LEA proteins. It is of interest that 71% of the sequences could be classified as novel, this may of course be a consequence of a deficiency of plant genes, in particular bryophyte genes, within the public databases but it may also indicate that there are genes involved in desiccation responses or tolerance that have yet to be isolated. This was underscored in a more extensive analysis of the rehydration transcriptome of *T. ruralis* that determined that 40.3% of the 5,563 clusters (contig groups representing individual genes) derived from an EST collection of 10,368 cDNA clones could not be assigned identity by comparison with sequences in all available public databases (Oliver *et al.*, 2004). The EST collection described by Oliver *et al.* (2004) was derived from a non-normalized rehydration specific library, non-normalized to allow for some assessment of transcript abundance during the initial two hours following rehydration. Genome ontology (GO) mapping of the *Tortula* clusters gave a broad look at what cellular activities appear to be emphasized in the rehydrated gametophytes and, as expected from our previous biochemical investigations, the protein synthetic machinery (both in structure and control), membrane structure and metabolism, and the need to reestablish or maintain plastid integrity were central to the response. The GO analysis also generated new directions and hypotheses to follow by indicating the prominence of membrane transport, phosphorylation and signal transduction, in the rehydration response to desiccation. Signal transduction is especially intriguing with regards to desiccation tolerance in *T. ruralis* given that translational controls appear more important in the alteration of gene expression than do alterations in transcriptional activities, although the rehydration cDNA library was derived from rehydrated rapid-dried gametophytes that do appear to rely on transcription to replenish the supply of rehydrin transcripts.

Of the top 30 most abundant transcripts reported for the rehydration transcriptome seven appear to encode LEA or LEA-like proteins (Table 1 and Oliver *et al.*, 2004). This led us to suggest that LEA proteins in *Tortula* may have had a role in protecting cellular constituents during rehydration as well as, or instead of, its generally assumed role of importance in protection during the drying process. The significance of this was amplified in an expression profile study using a cDNA microarray constructed from the 5,563 individual clus-

TABLE 1. *Abundant LEA-like protein transcripts in the* Tortula *rehydration library.*

#ESTs cluster	Description	Bit score	E-value
40	Q9XFD0 ABA-inducible protein WRAB1 (LEA/RAB-related COR)	63.93	2.88E-09
37	O16527 Caenorhabditis elegans CE-LEA	75.87	6.51E-13
32	Q9ZRF8 Hydrophobic LEA-like protein (Oryza sativa)	100.91	1.84E-20
28	P13934 Late embryogenesis abundant (LEA) protein 76 (B. napus)	51.60	1.42E-05
22	O16527 Caenorhabditis elegans CE-LEA	50.83	2.02E-05
21	Q9RV58 Protein DR1172-LEA type 1 family	55.45	1.01E-06
18	Q9LF88 Putative late embryogenesis abundant protein (Arabidopsis)	53.91	2.63E-06

Clusters are ordered by the number of individual ESTs that constitute a distinct cluster. Bit scores and E-values are derived from a BLASTx search of a customized database derived from UNIPROT as described in Oliver *et al.*, 2004.

ters derived from our EST collection. Twenty-four of the clusters that exhibited at least a two-fold increase in the accumulation of its corresponding transcripts in gametophytes that had been rehydrated for between 1 and 2 hr following rehydration have sequence similarity to known LEA protein sequences (Table 2). These transcripts are also elevated greater than two-fold in the polysomal RNA fraction indicating their recruitment into the translational mRNA pool. These data are consistent with the classification of these transcripts as rehydrins, and in fact one of the putative LEA like proteins whose transcript is elevated in these two fractions is the rehydrin Tr288, a rehydrin that we have described as a LEA-like protein (Velten and Oliver, 2001). Each cluster represents an independent nucleotide sequence even though several match the same LEA sequence from the public databases (indicative of the conserved nature of these proteins) which implies that there are several LEA proteins in *T. ruralis* that are available to the gametophytes following rehydration. If LEA proteins are preferentially synthesized in *T. ruralis* in response to rehydration, for which our evidence indicates but does not directly demonstrate, then it is reasonable to assume that LEA proteins play a role in cellular recovery in bryophytes as well as, or not, a role in cellular protection during dehydration. What the actual role of LEA proteins could be during the recovery from desiccation is yet to be determined but some clues have recently been uncovered that allow us to postulate a testable hypothesis. Koag *et al.* (2003) demonstrated that the maize DHN1, a group 2 LEA (Dehydrin), was capable of binding to lipid vesicles with some degree of specificity. The DHN1 protein preferred small vesicles to larger ones and vesicles that contained a significant proportion of acidic phospholipids. In addition, in binding to the vesicles, the protein was determined to undergo a conformational change resulting in an increase in alpha-helicity within

TABLE 2. *Expression profile of LEA-like transcripts in the* Tortula ruralis *EST collection.*

Tortula ID	Gene ID	Total REH	Poly REH
Cluster_1809	Q8S7U3 Putative LEA protein	7.5	3.4
Cluster_3490	Q9XES7 Seed maturation protein	7.2	6.6
Cluster_42	LEA3MAIZE LEA protein	6.3	6.0
Cluster_392	Q9ZQW6 Lea protein	5.7	2.1
Cluster_319	O16527 CE-LEA	5.3	3.0
Cluster_1646	Q9LJ97 Seed maturation protein	4.7	4.6
Cluster_136	LEA1HORVU (P14928)	4.6	4.5
Cluster_147	Q39801 51kDa seed maturation protein	4.5	2.8
Cluster_861	Q9LJ97 Seed maturation protein	4.4	3.7
Cluster_41	O16527 CE-LEA	4.4	2.9
Cluster_28	LEA1HORVU (P14928)	4.4	5.4
Cluster_41	O16527 CE-LEA	4.2	3.5
Cluster_596	O16527 CE-LEA	4.2	2.1
Cluster_139	Q9FKV7 LEA protein-like	4.2	4.7
Cluster_36	LEA3MAIZE LEA protein	3.8	3.9
Cluster_1647	Q8GWT7 Putative LEA	3.7	4.2
Cluster_1409	Q9LF88 Putative LEA protein	3.4	2.0
Cluster_2300	Q9XES7 Seed maturation protein	3.2	2.7
Cluster_40	LEA1HORVU (P14928)	3.2	2.7
Cluster_2020	O04371 Rehydrin Tr288	3.0	2.1
Cluster_935	Q39660 LEA protein homolog	2.9	2.1
Cluster_144	O16527 CE-LEA	2.8	2.4
Cluster_935	Q39660 LEA protein homolog	2.5	2.3
Cluster_578	Q8GV49 LEA1 protein	2.3	2.5

Total and polysomal RNA was isolated from rapid-dried rehydrated 1–2 hr (treated) and hydrated (control) gametophytes. Three biological replicates from each condition were probed with the *Tortula* cDNA array in a triple dye-swap experiment for a total of six hybridizations per condition. Data are expressed as mean ratios (rehydrated/hydrated). A total of 24 LEA-like transcripts were up-regulated more than two-fold ($P \leq 0.05$) in response to a dehydration-rehydration event in both the total and polysomal RNA fractions.

the protein secondary structure. The authors suggest dehydrins may undergo a function-related conformational change at the water-membrane interface that may aid in the stabilization of small lipid vesicles or other endomembrane structures within cells. In collaboration with Julia George, at the University of Illinois, we have similar data using the Tr288 LEA-like protein from *T. ruralis*. Tr288 has no sequence similarity, nucleotide or amino-acid, to any other plant protein including the LEA proteins with the exception of a single putative ''K-Box'' motif, a signature motif of angiosperm dehydrins, near the carboxy-terminal of the Tr288 protein (Velten and Oliver, 2001). However, Tr288 is structurally similar to the LEA protein family in all other respects. It is highly hydrophilic, glycine-rich (19.6%), and contains 15 copies of a conserved amino acid sequence motif that is predicted to form amphipathic alpha helices. Purified protein, derived from expression of a Tr288 construct in *E. coli*, preferentially binds to lipid vesicles that are composed of a 3:1 mixture of phosphotidylcholine and phosphatidic acid. If the vesicles are constructed of phosphotidylcholine alone, Tr288 appears to partially solubilize the lipid (unpublished data). Although we have yet to demonstrate any alteration in the conformation of the protein as it interacts with the lipid vesicles or any *in vivo* confirmation of this activity it does appear that at least some LEA proteins may function as lipid stabilizing proteins. This leads us to the hypothesis that in rehydrating *T. ruralis* gametophytes LEA proteins may serve a role in stabilizing membranes, or perhaps lipid transport for reconstitution of damage membranes, during the cellular upheaval that results from the unfettered inrush of water during rehydration.

PHYLOGENETIC RELATIONSHIPS OF TR288

The apparent emphasis on the synthesis of LEA proteins during the initial periods following rehydration of dried gametophytes and the possibility that they play a role in membrane stability or reconstitution suggests that these genes may play a key adaptive role in the evolution of desiccation tolerance in plants. In order to gain some insight into this possibility, we utilized the conserved nature of the nucleotide sequences corresponding to the 15 amino-acid repeats within the Tr288 gene to develop a PCR-based strategy to identify possible Tr288 orthologs in a wide range of land plant species. The purpose of such an investigation was to determine the evolutionary relationship of the Tr288 gene to desiccation tolerance. This approach is limited by the ability of the PCR primers to allow amplification of a fragment from the ortholog and any conclusions that are drawn are done so under the caveat that the absence of a PCR fragment may simply mean that the primer was incapable of allowing extension of a DNA product because of a mismatch at the 3′ end. To alleviate this problem, at least in part, we chose the most highly conserved sequence within the repeats for the 3′ portion of the primers and cloned and sequenced each PCR product to be sure that it

FIG. 1. Mapping of Tr288 orthologs to an unrooted phylogenetic network of a basal portion of land plant phylogeny (synthesized from several previous studies, published and unpublished). Those genera whose names are in italics have been classified as desiccation-tolerant according to reports in the literature, see Proctor and Pence (2002), and from unpublished observation (Oliver and Mishler).

contained sequence similar to that of the *T. ruralis* Tr288. The location of the PCR primers within the conserved portion of the repeats increased our potential for isolating an othologous sequences because the primers, if there are more than two repeats, will generate several possible PCR fragments rather than just one and if each is cloned and sequenced will generate a better sequence comparison for the ortholog assessment.

The results of this analysis were mapped onto a phylogenetic tree, synthesized from previous studies, to gain an insight into the evolutionary history of this gene (Fig. 1). On this tree, Tr288 orthologs appear to correlate well with desiccation tolerance within the bryophyte taxa, with the exception of the tropical desiccation-tolerant members of the *Exostratum*, *Octoblepharum*, *Leucophanes*, and *Anthrocormus* genera (even though *Mitthyridium*, a related genus, does have a Tr288 ortholog). This raises the interesting possibility that these tropical mosses that dry under elevated temperatures may have a mechanism of tolerance that differs from that seen in more temperate species and one that has lost the need for a Tr288 homolog or has evolved a more efficient form of this type of protein. Of even more significance is that a Tr288 ortholog is completely absent from the liverworts even though many are desiccation-tolerant. This may be a consequence of the fact that liverworts branched off earliest in land plant evolution and that the ancestral form of the Tr288 gene either had yet to evolve into a structure that would allow detection by our PCR approach, or that these primitive plants may actually have a different strategy and mechanism of desiccation tolerance.

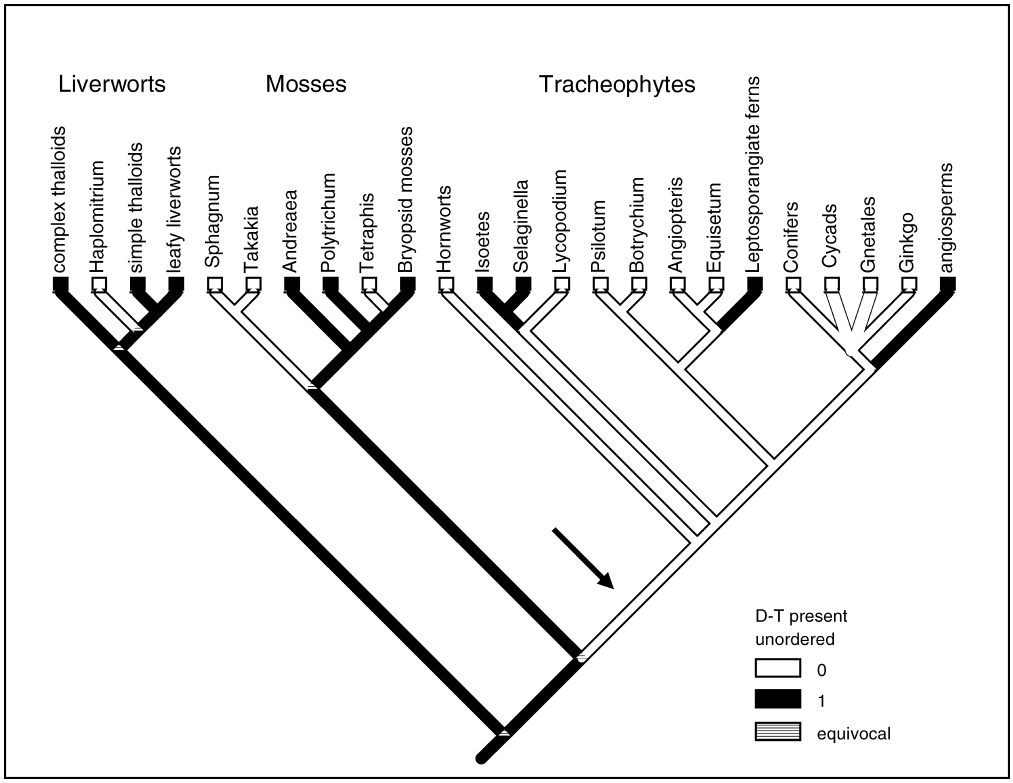

FIG. 2. Simplified phylogeny of the land plants, showing the reconstruction of presence of vegetative desiccation tolerance (in at least some members of indicated clade) in black, non-tolerance in white, and equivocal reconstruction by hatching. The arrow marks where the ancestral condition can unequivocally be reconstructed as non-tolerant. Branching topology based on Shaw and Renzaglia, 2004; Pryer *et al.*, 2004; Kelch *et al.*, 2004; distribution of desiccation-tolerance based on Wood and Peng (2005) and Proctor and Pence, 2002.

Tr288 orthologs are still present in the tracheophytes. This could mean that the gene has been co-opted into a different role, perhaps still involved in dehydration tolerance mechanisms or possibly involved in propagule desiccation tolerance, or has become simply an inactive gene remnant. Although all of these possibilities are simply speculative this analysis has opened up some interesting hypotheses and research directions to be tested and followed. This study has also led us to re-evaluate what we understand of the evolution of desiccation tolerance in the land plants.

PHYLOGENETIC INFERENCES REVISITED

An evolutionary scenario for the evolution of vegetative desiccation tolerance was first given by Oliver *et al.* (2000), and presented there as a testable hypothesis. Because of the widespread occurrence of vegetative desiccation tolerance in the bryophytes (the earliest diverging living clades of land plants), they argued that it is likely that it was primitively present in the land plants. They further hypothesized that vegetative desiccation tolerance was subsequently lost in the early evolution of tracheophytes and then re-evolved multiple times in several separate tracheophyte lineages. Their postulated mechanistic explanation for this pattern can be summarized as follows: the primitive mechanism of tolerance exhibited by the first plants probably involved a constitutive level of cellular protection coupled with an efficient and active repair process, similar to what we have described above for modern-day desiccation-tolerant bryophytes. This desiccation tolerance came at a cost, because metabolic rates are low in tolerant plants as compared to plants that do not maintain costly mechanisms for tolerance. As plants evolved to fill the various niches available to them on dry land, loss of tolerance was favored because of the internalization of water relations as the vascular plants became larger and more complex. Genes that had originally evolved for vegetative cellular protection and repair were possibly recruited for different but related processes such as the response to water stress and, more important, the desiccation tolerance of reproductive propagules (seeds). In turn, Oliver *et al.* (2000) speculated that once established in seeds, the developmentally induced cellular protection system became available for induction in vegetative tissues by environmental cues that are related to drying. This may have led to an evolutionarily more recent vegetative desiccation tolerance mechanism, evolved from that programmed into seed development, as certain phylogenetically scattered lineages of angiosperms spread into very arid environments.

This series of hypotheses needs to be tested in light of more recent phylogenetic knowledge, and greater knowledge of the distribution of desiccation tolerance in land plants. Figure 2 shows a simplified picture of

the overall phylogenetic distribution of desiccation tolerance in the current phylogeny of land plants. It is evident from this figure that the basal condition for the land plants has now become equivocal, given this more detailed picture. It makes sense, given the arguments in the earlier part of this paper, that vegetative desiccation tolerance is likely to have been present in the earliest land plants. However, it is difficult to demonstrate this formally with character reconstructions at present. Part of the problem comes from the lack of knowledge about desiccation tolerance in the outgroups, the close ''green algal'' relatives of land plants (*Chara, Coleochaete*, and relatives; Lewis and McCourt, 2004). Also, there are key groups near the base of liverwort and moss phylogenies that do not appear to be desiccation-tolerant (although these need to be surveyed in greater detail). It has been a long time since the branches near the base of this tree diverged from each other (450–500 MYA), extinction has taken its toll, and many of the modern taxa (such as Sphagnum) are highly modified in structure and physiology from their ancestral conditions. To resolve this character reconstruction question, better sampling for desiccation tolerance is needed, especially among the basal lineages. It is also likely that if highly derived taxa such as *Sphagnum* were once desiccation-tolerant, but lost the phenotype, telltale signs will remain in their genomes such as the presence of rehydrins that can be discovered by comparative genomics. So there is hope of resolving this important ancestral reconstruction issue someday.

The possibility that vegetative desiccation tolerance may not have been present at the very bottom of the land plant phylogenetic tree suggests that it may be necessary to expand on the hypothesis given in Oliver *et al.*, 2000. Vegetative desiccation tolerance is certainly not the full story. It is quite possible that an even more primitive condition in precursors to land plants is desiccation-tolerant spores. Perhaps the earliest manifestation of desiccation tolerance was in the spores of land plants (and probably their charophyte alga relatives), and then desiccation tolerance became expressed in vegetative tissues in early land plants. As previously postulated by Oliver *et al.* (2000), expression appears to have been lost in the vegetative tissues of the early tracheophytes, yet regained in the some widely separated lineages. We don't know what happened in spores, however—it is quite possible that desiccation tolerance was widely retained throughout in the spores of tracheophytes. Very little is known about desiccation tolerance in spores, and it is imperative that this be studied soon in a breadth of taxa to test this expanded hypothesis.

It is clear from Figure 2, that vegetative desiccation tolerance was ancestrally lacking in the tracheophytes (starting from the branch shown with arrow). The number of times it was regained is still not certain, given the lack of complete surveys for the presence of desiccation tolerance. However, recently increased resolution of the angiosperm phylogeny allows a clearer picture of the distribution of the unusual phenotype of desiccation tolerance in this most diverse group of land plants. Using the online phylogenetic trees at the Angiosperm Phylogeny Website (Stevens, 2001), and mapping on the most recent survey of the occurrence of desiccation tolerance (Proctor and Pence, 2002), allows the following insights: (1) within the angiosperms, at least ten phylogenetically independent cases of re-evolution of desiccation tolerance occurred; (2) at least four of these cases arose in the monocots, and probably more in the large families Poaceae and Cyperaceae; (3) no cases occur in basal dicots, but six occur in the Eudicots; (4) Four cases occur in the Asterids, but interestingly there are none known so far in the Rosids. There will certainly be more cases found as more angiosperms are studied, and it will be interesting to see what habitat and phylogenetic correlations are uncovered.

ACKNOWLEDGMENTS

We thank Peter Alpert for putting in the lion's share of the work organizing the symposium, Michael Proctor and one anonymous reviewer provided helpful comments. We also thank Sarah Learmonth for her expert technical help. Work presented here was supported in part by USDA-ARS CRIS project 6208-21000-012-00 (MJO and JV) and by NSF grants DEB-9712347 (the Deep Gene RCN) and DEB-0228729 (the Green Tree of Life project) to BDM. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

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