Metabolomic Profiling in *Selaginella lepidophylla* at Various Hydration States Provides New Insights into the Mechanistic Basis of Desiccation Tolerance

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**ABSTRACT** *Selaginella lepidophylla* is one of only a few species of spike mosses (*Selaginellaceae*) that have evolved desiccation tolerance (DT) or the ability to ‘resurrect’ from an air-dried state. In order to understand the metabolic basis of DT, *S. lepidophylla* was subjected to a five-stage, rehydration/dehydration cycle, then analyzed using non-biased, global metabolomics profiling technology based on GC/MS and UHLC/MS/MS platforms. A total of 251 metabolites including 167 named (66.5%) and 84 (33.4%) unnamed compounds were characterized. Only 42 (16.7%) and 74 (29.5%) of compounds showed significantly increased or decreased abundance, respectively, indicating that most compounds were produced constitutively, including highly abundant trehalose, sucrose, and glucose. Several glycolysis/gluconeogenesis and tricarboxylic acid (TCA) cycle intermediates showed increased abundance at 100% relative water content (RWC) and 50% RWC. Vanillate, a potent antioxidant, was also more abundant in the hydrated state. Many different sugar alcohols and sugar acids were more abundant in the hydrated state. These polyols likely decelerate the rate of water loss during the drying process as well as slow water absorption during rehydration, stabilize proteins, and scavenge reactive oxygen species (ROS). In contrast, nitrogen-rich and γ-glutamyl amino acids, citrulline, and nucleotide catabolism products (e.g. allantoin) were more abundant in the dry states, suggesting that these compounds might play important roles in nitrogen remobilization during rehydration or in ROS scavenging. UV-protective compounds such as 3-(3-hydroxyphenyl)propionate, apigenin, and naringenin, were more abundant in the dry states. Most lipids were produced constitutively, with the exception of choline phosphate, which was more abundant in dry states and likely plays a role in membrane hydration and stabilization. In contrast, several polyunsaturated fatty acids were more abundant in the hydrated states, suggesting that these compounds likely help maintain membrane fluidity during dehydration. Lastly, *S. lepidophylla* contained seven unnamed compounds that displayed twofold or greater abundance in dry or rehydrating states, suggesting that these compounds might play adaptive roles in DT.

Key words: abiotic/environmental stress; mass spectrometry; metabolomics; oxidative/photooxidative stress; desiccation tolerance; *Selaginella*.

**INTRODUCTION**

Lycophytes represent a key vascular plant lineage that includes the Lycopodiaceae (club mosses), the Isoetaceae (quillworts), and the Selaginellaceae (spike mosses) that arose over 400 million years ago during the Silurian and dominated the Earth’s flora from the Devonian through the Carboniferous to the end of the Permian (Friedman, 2011). Dramatic decreases in atmospheric CO₂ concentrations occurred during these Paleozoic epochs and the resulting decomposed flora now provide a major source of energy to mankind in the form of coal (Beerling, 2002; Gensel, 2008). Today, extant lycophytes comprise a single monophyletic clade of just over 1000 species.
Within the spike mosses, the single genus *Selaginella* contains about 700 species that now exploit a diverse array of arctic, temperate, tropical, and semi-arid habitats (Banks, 2009). Although most spike moss species are susceptible to desiccation, a few species have evolved the ability to survive vegetative tissue drying, defined as the near complete loss (80%–95%) of protoplasmic water (Tuba et al., 1998), and referred to as desiccation tolerance (DT) (Oliver et al., 2000a). Such species include *S. lepidophylla* (Iturriaga et al., 2006), *S. bryopteris* (Deeba et al., 2009), and *S. tamariscina* (Wang et al., 2010). The DT trait is relatively common in algae, lichens, and mosses (Alpert, 2006; Wood, 2007). However, among vascular plants, DT is extremely rare; only about ~0.15% of species being known as resurrection plants (Oliver et al., 2000a; Porembski and Barthlott, 2000; Proctor and Pence, 2002; Proctor and Tuba, 2002).

In terms of response to desiccation, resurrection mosses (e.g., bryophytes) rely on a combination of constitutive protection and inducible repair mechanisms that permit dehydration to occur within minutes while maintaining their photosynthetic apparatus relatively intact (Tuba et al., 1998; Oliver et al., 2000b, 2005). In contrast, angiosperms require a much longer time to dehydrate and still remain viable, presumably to allow sufficient time for mobilizing adaptive responses and accumulation of key metabolites, such as sucrose, to survive in the desiccated state (Bernachia et al., 1996; Oliver et al., 2000a). In contrast, lycophytes represent a plant lineage that lies between mosses and angiosperms (Oliver et al., 2000a) and thus might be expected to exhibit both constitutive and inducible adaptive mechanisms of DT. Many monocot DT species lose their photosynthetic apparatus, at least partially, during the dehydration process (Farrant, 1996; Oliver et al., 2000b, 2005). In the current study, a total of 251 metabolites were identified to provide insights into both constitutive and inducible metabolic strategies to survive and recover from desiccation of vegetative tissues.

**RESULTS AND DISCUSSION**

Metabolomics approaches can provide a detailed understanding of an organism’s phenotype (Schauer and Fernie, 2006; Cascante and Marin, 2008), and disposition towards and response to environmental stresses (Sanchez et al., 2008; Shulaev et al., 2008; Urano et al., 2010). Recent studies have compared two closely related species of *Sporobolus* (Oliver et al., 2011) and *Selaginella* (Yobi et al., 2012) in order to discern key metabolic differences that might contribute to DT. In the current study, a total of 251 metabolites were identified to provide insights into both constitutive and inducible metabolite abundance patterns essential for the acquisition of DT in the resurrection lycophyte, *S. lepidophylla*.
**S. lepidophylla and Dehydration**

In order to discern relative metabolite abundance changes in *S. lepidophylla* during a rehydration/dehydration cycle, plants were allowed to hydrate, then dehydrate, and their RWC was tracked over a 24-h period. Upon hydration, water uptake was relatively rapid; 70% RWC was achieved within 1 h and 100% RWC after 24 h (Figure 1A). When hydrated, *S. lepidophylla* plants displayed fully expanded green microphylls (Figure 1B). In contrast, water loss during dehydration was markedly slower, with 70% water loss taking more than 4 h and the plants retaining 7% RWC after 24 h of drying. The rate of water loss in *S. lepidophylla* was similar to *S. bryopteris*, a related DT species, which required about 6 h to lose 80%–90% of its RWC (Deeba et al., 2009; Pandey et al., 2010). During dehydration, the microphylls curl to form a tight ball (Figure 1B), which is an adaptive response to protect the plant against damage due to high irradiance or temperatures, or both (Lebkuecher and Eickmeier, 1991, 1992, 1993). Chlorophyll is maintained at least partially on the inner surface of the microphylls, indicating that *S. lepidophylla* is homiochlorophyllous (Tuba et al., 1998).

**Metabolome Composition of S. lepidophylla**

To assess the metabolomic response of *S. lepidophylla* to a rehydration/dehydration cycle, six biological replicate tissue samples were collected from plants sampled at full dehydration 1 (7% RWC, DRY-1), partial dehydration (50% RWC, DEH-50), full rehydration (100% RWC, HYD), partial dehydration (50% RWC, DEH-50), and full dehydration 2 (7% RWC, DRY-2), and analyzed using non-biased, global metabolome technology based on GC/MS and UHLC/MS/MS² platforms (Evans et al., 2009). The combined platforms detected a total of 251 metabolites, of which 167 (66.5%) were named and 84 (33.4%) were unnamed (Figure 2 and Supplemental Table 1). After the named metabolites were mapped onto general biochemical pathways, they were categorized into classes of which amino acids were the most prevalent (19%), followed by carbohydrates (16%), lipids (13%), cofactors (6%), nucleotides (5%), peptides (4%), and secondary metabolites (3%). Lastly, unnamed compounds in *S. lepidophylla* accounted for one-third of all compounds surveyed (Figure 2).

**Metabolic Differences during a Rehydration/Dehydration Cycle**

Partial Least Squares-Discriminant Analysis (PLS-DA) was used to build a comparative model of the five different hydration states in *S. lepidophylla*. Metabolites missing three or more out of six (50%) biological replicate values were excluded, resulting in a total of 204 metabolites for comparison. The first three PLS-DA components explained 52.2% of the variation ($R^2 = 0.50$; $Q^2 = 0.40$) and showed distinct clustering among each of the five states, suggesting that various metabolites likely accounted for the differences observed in the model (Figure 3). The metabolomes of the two dry states...
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at 7% RWC were similar to one another. The metabolic profile of the fully hydrated state at 100% RWC was distinct from these dry states, and that of the 50% RWC rehydration state was intermediate between the dry and fully hydrated plants. Interestingly, the 50% RWC dehydration state was also clearly distinct from those of the 50% RWC rehydration and the initial dry states, suggesting that distinct metabolic pathways operate during rehydration compared with dehydration.

In comparing the S. lepidophylla metabolite profiles across all five hydration states, a total of 114 (45.4%) compounds were detected that showed significantly altered abundance ($p < 0.05$), including 42 (16.7%, 27 named and 15 unnamed) that were more abundant in one or more of the dry or partially dry states, and 74 (29.5%, 57 named and 17 unnamed) that showed a greater abundance in the fully hydrated state. Two metabolites (i.e. pyruvate, galactose) showed variable abundance patterns (Supplemental Table 1). A total of 11 metabolites were significantly ($p < 0.05$) more abundant in both dry states compared with the fully hydrated state. In addition, 11 metabolites were more abundant only in the initial dry state, whereas the abundance of only one compound was higher in the second dry state. The precise origin of these differences remains unclear, but might be due to differential metabolic activity changes that occur between the initial and subsequent rehydration/dehydration cycles.

In contrast, comparing the hydrated state with the two fully dry states, 26 metabolites showed significantly ($p < 0.05$) greater abundance than in both dry states. An additional 46 compounds were significantly ($p < 0.05$) more abundant when compared with the second dry state only. Although identical conditions were used for each dehydration episode, these differences between the two dry states might be due to variation arising from repeated hydration and dehydration cycles associated with the life history of different sets of plants.

Amino acids, peptides, and nucleotide metabolites were notably overrepresented in the dry states (Supplemental Table 1). In contrast, various carbohydrates, particularly 4C, 5C, and 6C sugars and sugar alcohols, lipids, or lipid metabolites (with the exception of choline phosphate), and cofactors were clearly overrepresented in the hydrated state (Supplemental Table 1).

Carbohydrates and Markers of Energy Metabolism

Many differences were observed in the abundance of metabolites of glycolysis/gluconeogenesis and the tricarboxylic acid (TCA) cycle. For example, glucose-6-phosphate, fructose-6-phosphate, maltose-6-phosphate, glyceraldehyde, and pyruvate showed a significantly ($p < 0.05$) increase and peak abundance during dehydration (DEH-50) (Figure 4). Several TCA cycle intermediates, such as oxaloacetate, fumarate, succinate, and alpha-ketoglutarate, also showed peak abundance during dehydration, whereas succinate was more abundant in the hydrated state (Figure 4 and Supplemental Table 1). Similarly, glyceraldehyde, lactate, and pyruvate were significantly ($p < 0.05$) more abundant in the hydrated state, with the exception of the DEH-50 state (Supplemental Table 1). The peak accumulation of many glycolysis/gluconeogenesis metabolites suggests that metabolic flux through these pathways is essential for the production of downstream products important for the acquisition of DT.

Various oligosaccharides accumulate in response to drying in the vegetative tissues of all DT tracheophytes studied to date (Alpert and Oliver, 2002). Based on the total relative ion counts, trehalose, sucrose, and glucose were the most abundant compounds within S. lepidophylla tissues and accounted for an estimated 50% of the total metabolites. These compounds tended to be more abundant in the hydrated state relative to the dry state(s) (Supplemental Table 1) and their relative abundances were trehalose > sucrose > glucose. Sucrose alone, or in combination with other sugars, such as raffinose-series oligosaccharides and cyclitols, or with macromolecules, such as late embryogenesis abundant (LEA) proteins (Wolkers et al., 2001; Shimizu et al., 2010), likely protects vegetative tissues of resurrection species upon drying through the formation of anhydrous glass or by replacement of water to prevent damaging interactions, such as membrane fusion (Hoekstra et al., 2001). S. lepidophylla synthesizes high constitutive concentrations of sucrose (and trehalose and glucose), and thus resembles DT mosses that maintain constitutively high sucrose levels that do not further increase during drying (Smirnoff, 1992). In contrast, in a DT fern (Farrant et al., 2009) and all angiosperm species examined to date, sucrose accumulation is induced following the imposition of dehydration stress (Ghasempour et al., 1998; Whittaker et al., 2001; Peters et al., 2007; Toldi et al., 2009; Oliver et al., 2011).

Figure 3. Metabolites at Different Hydration States in S. lepidophylla as Defined by Partial Least-Squares-Discriminant Analysis (PLS-DA) Constructed from 204 Metabolites.

The plot shows the three components that explains 52.2% of the metabolite signatures ($R^2 = 0.50$; $Q^2 = 0.40$). Upside-down triangles (red) represent initial dry state (DRY-1), squares (brown) represent 50% rehydration (REH-50), asterisks (green) represent fully hydrated (HYD), crosses (cyan) represent 50% dehydrated (DEH-50), and diamonds (moss green) represent the second dry state (DRY-2).
Trehalose, a non-reducing disaccharide, is known to accumulate in high amounts in lycophytes (Adams et al., 1990; Iturriaga et al., 2000; Liu et al., 2008) as well as in many lower-order DT organisms (Elbein et al., 2003). However, a critical role for this disaccharide in DT is questionable for several reasons. First, several resurrection ferns and angiosperms accumulate trehalose in only very small amounts (Ghasempour et al., 1998; Farrant et al., 2009). Second, a Saccharomyces cerevisiae mutant defective in trehalose biosynthesis can maintain DT, indicating that trehalose is neither necessary nor sufficient for DT (Ratnakumar and Tunnacliffe, 2006). However, trehalose has been shown to act synergistically with LEA proteins to stabilize client enzymes by reducing their aggregation (Goyal et al., 2005). While trehalose alone might not be sufficient for DT, it might act cooperatively, as do other sugars, as a chemical chaperone to enhance the molecular shield function of LEA proteins in reducing desiccation-induced aggregation of proteins (Chakrabortee et al., 2012).

Interestingly, the osmoprotectant glucosyglycerol (2-O-alpha-D-glucopyranosyl-sn-glycerol) was also detected in S. lepidophylla and was more abundant in the hydrated state relative to the dry state(s) (Supplemental Table 1). Glucosyglycerol is typically found in cyanobacteria (Klähn and Hagemann, 2011). This compatible solute can stabilize various enzymes against thermal denaturation as well as improve enzyme stability eightfold following lyophilization and rehydration (Sawangwan et al., 2010). The constitutive and concerted production of sucrose, trehalose, and glucosyglycerol are very likely critical to the preservation of diverse enzyme activities during and following desiccation.

Sugar Alcohol Metabolism
Sugar alcohols or polyols are known to play important roles in water-deficit and salinity-stress adaptation and tolerance (Nuccio et al., 1999; Chen and Murata, 2002; Rontein et al.,

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**Figure 4.** Metabolites Associated with Energy Metabolism (Glycolysis and TCA Cycle) in S. lepidophylla Profiled during a Rehydration/Dehydration Cycle.

Compounds in red indicate greater relative abundance in one or more dry states. Compounds in green indicate greater relative abundance in the hydrated state. Dotted arrows indicate a gap in the pathway and double-headed arrows indicate a reversible reaction. The x-axis represents the percentage of relative water content (% RWC): initial dry state (DRY-1); 50% rehydrated (REH-50); fully rehydrated (HYD); 50% dehydrated (DEH-50); and second dry state (DRY-2). The y-axis box plots indicate the scaled intensity mean (closed triangles) and median (——) values, top/bottom ranges of boxes indicate upper/lower quartiles, respectively, and top/bottom whiskers indicate the maximum/minimum distribution of the data, respectively. Open circles indicate extreme data points. The p- and q-values for the comparisons are presented in Supplemental Table 1. G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; M-6-P, mannose-6-phosphate; 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate.
A variety of polyols showed significant ($p < 0.05$) changes in abundance in *S. lepidophylla* undergoing rehydration/dehydration, with peak accumulation occurring in the fully hydrated state (Figure 5 and Supplemental Table 1). For example, xylitol abundance was 10–33-fold greater at 100% RWC compared with the DRY-1 and DRY-2 states, respectively (Figure 5). Relative amounts of other polyols were also significantly higher in the fully hydrated state: ribitol was 16.5–20.0-fold higher, sorbitol was 5.8–20.0-fold higher, erythritol was 3.3–9.0-fold higher, compared with the DRY-1 and DRY-2 states, respectively, and glycerol was 1.4–2.6-fold higher compared with the DRY-1 and DRY-2 states, respectively, and 5.0-fold higher in the REH-50 state. Threitol was 3.0–5.0-fold higher compared with the DRY-1 and DRY-2 states, respectively; however, these differences were not significant. In addition, related oxidation products of these sugars included gluconate and erythrionate, which were 2.8–16.5-fold and 2.0–2.5-fold more abundant, respectively, in HYD compared with the DRY-1 and DRY-2 states. Lastly, N-acetylg glucosamine, a monosaccharide derived from glucose and an important structural sugar, was 2.0-fold more abundant in HYD compared with the dried states (Figure 5).

In contrast, mannitol and galactose were significantly ($p < 0.05$) more abundant in the DRY-1 state (2.0-fold and 1.9-fold, respectively) compared with the fully hydrated state (Supplemental Table 1). In addition to its probable role as an osmolyte, mannitol has been shown to act as a hydroxyl radical scavenger (Shen et al., 1999). Inositol 1-phosphate was 1.2–1.8-fold more abundant in all dehydration states compared with the fully hydrated state, but these differences were not significant (Supplemental Table 1). In contrast to other DT species (Farrant et al., 2009; Oliver et al., 2011), raffinose-series sugars (e.g. galactinol, raffinose, stachyose) were not detected and probably do not participate in DT in *S. lepidophylla*. Investigation into the metabolomes of other lycophytes is needed to determine whether this observation holds true for all members of this ancient order within the plant kingdom. Raffinose-series sugars have been shown to act as ROS scavengers in *Arabidopsis* (Nishizawa et al., 2008; Nishizawa-Yokoi et al., 2008). The lack of detectable raffinose-series sugars in *S. lepidophylla* indicates that this function might be fulfilled by other sugars or other diverse compounds with ROS-scavenging activities.

The accumulation of polyols in the fully hydrated state in *S. lepidophylla* indicates several important roles for these sugars. Because polyols, especially glycerol, reduce the surface tension of water (Tiwari and Bhat, 2006) and display strong water-binding activity via hydrogen bonding (Kataoka et al., 2011; Weng et al., 2011), they might decelerate the rate of water loss during the drying process (Figure 1A). Indeed, the ability of *S. stapfianus* to reduce the rate of water loss during dehydration, compared to the DS *S. pyramidalis*, appears to be a result of a similar metabolic ‘priming’ of the hydrated tissue with osmolytes (Oliver et al., 2011). Slowing the rate of water loss might permit more time for the biosynthesis of desiccation-adaptive proteins and metabolites that are required for the establishment of DT (Alpert and Oliver, 2002). Conversely, biosynthesis of polyols might also slow the rate of water absorption during rehydration. Notably, several polyols such as sorbitol and erythritol exhibited increased accumulation at the 50% RWC rehydration stage (REH-50), consistent with this hypothesis. A direct comparison of DT *S. lepidophylla* and DS *S. moellendorfii* indicated that the DT species accumulated significantly greater amounts of no fewer than seven different polyols in the fully hydrated state (Yobi et al., 2012). Furthermore, a recent comparison of DT *Sporobolus stapfianus* and DS *S. pyramidalis* showed that several polyols, including arabitol, erythritol, and mannotol, were several-fold more abundant in fully hydrated leaves of the DT species (Oliver et al., 2011). These observations suggest that constitutive expression of polyols in the hydrated state might be critical for many resurrection species to slow the rate of water loss during dehydration as well as water uptake during rehydration. Indeed, *S. lepidophylla* shows a slower rate of rehydration than does *S. moellendorfii* (Yobi et al., 2012).

In addition to a role in water equilibrium, several polyols can also act as osmoprotectants by stabilizing protein (yeast hexokinase A) structure against thermal (Tiwari and Bhat, 2006) or acidic (Devaraneni et al., 2012) denaturation. Glycerol, sorbitol, and xylitol exhibit better stabilization than glucose or trehalose through preferential hydration of proteins in their denatured state (Xie and Timasheff, 1997) by increasing surface tension (Kaushik and Bhat, 1998). Both sorbitol and xylitol showed the greatest relative abundance in *S. lepidophylla* compared with *S. moellendorfii* (Yobi et al., 2012) and in this study (Figure 5), which is consistent with such a protein-stabilizing function. Several sugar alcohols, such as myo-inositol, mannotol, and sorbitol, also possess the ability to scavenge hydroxyl radicals as a way of reducing oxidative damage (Sanchez et al., 2008). Thus, polyol accumulation might limit the oxidative stress burden of DT plants during the early phases of dehydration and rehydration. In addition to osmotic adjustment, the accumulation of polyols might also facilitate other metabolic functions such as consumption of NADH, which might enhance redox control and reduce ROS production in the cells (Shen et al., 1999). Lastly, sugar alcohols might stimulate the expression of stress-adaptive genes, as has been observed in *Arabidopsis* plants engineered to express mannitol (Chan et al., 2011).

### Amino Acid and Peptide Metabolism

Of the 48 amino acids and derivatives detected, relative amounts of 10 of them occurred in significantly ($p < 0.05$) greater amounts in one or more of the dry states than in the fully hydrated state, whereas only eight amino acids were
significantly ($p < 0.05$) more abundant in the hydrated state compared with one or more of the dry states (Supplemental Table 1). Those more abundant in a dry state included nitrogen-rich amino acids such as glutamine, glutamate, arginine, aspartate, citrulline (Figure 6), asparagine, 3-(3-hydroxyphenyl)propionate, N-6-trimethyllysine, trans-4-hydroxyproline, and ophthalmate (Supplemental Table 1). In contrast, glycine, quinate, and alanine were significantly ($p < 0.05$) more abundant in fully hydrated $S. \text{lepidophylla}$ compared with both dry and REH-50 states (Supplemental Table 1). In comparison, quinate accumulation was induced by dehydration (at 60% RWC) in DT $S. \text{stapfianus}$, but not in DS $S. \text{pyramidalis}$ (Oliver et al., 2011).

The accumulation of nitrogen-rich amino acids (e.g. asparagine, aspartate, arginine, glutamate, and glutamine) partially resembles the desiccation response of the DT grass $S. \text{stapfianus}$, in which several nitrogen-rich amino acids (e.g. asparagine, arginine, glutamine) accumulated to significantly greater concentrations in nearly dry or dry states (Martinelli et al., 2007; Oliver et al., 2011). This increased accumulation of nitrogen-rich amino acids might reflect an adaptation to the nitrogen-limiting conditions typically encountered by $S. \text{stapfianus}$ and other DT species in their natural habitat of rocky outcrops with nitrogen-poor soils (Burke, 2002). Alternatively, these amino acids might provide a nitrogen reservoir useful during the early stages of rehydration before the recovery of photosynthetic activity (Martinelli et al., 2007). In addition, these accumulated amino acids could provide precursors for the production of protective nitrogenous compounds, such as the antioxidant glutathione (see Figure 7).

Citrulline, a structural analog of arginine, was the only amino acid that was significantly ($p < 0.05$) more abundant in all dry and partially dry states compared with the hydrated state (Figure 6). This non-protein amino acid is thought to function either as a nitrogen reserve or as a hydroxyl radical scavenger, or both, and accumulates along with arginine, glutamate, and glutamine in response to drought stress in a drought-tolerant wild watermelon (Kawasaki et al., 2000; Akashi et al., 2001). Thus, citrulline might play similar roles in $S. \text{lepidophylla}$.

The amino acid derivative 3-(3-hydroxyphenyl)propionate was also more abundant in all dry and partially dry states.
compared with the hydrated state, and significantly so in the DRY-1 and DRY-2 states (Supplemental Table 1). Notably, the structure of 3-(3-hydroxyphenyl)propionate suggests that it might play a protective role against UV-light damage because closely related phenolic compounds, such as 3,4′-dihydroxypropiophenone-3-glucoside and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-β-d-glucoside, serve as UV-B absorbing sunscreens or filters in plants (Tegelberg et al., 2002) and human eye lenses (Bova et al., 1999), respectively. Additional research into the potential UV-protective effects of this compound is warranted.

N-6-trimethyllysine is a biosynthetic precursor of carnitine (Hoppel et al., 1980), a known osmoprotectant in *Escherichia coli* (Cánovas et al., 2007; Verheul et al., 1998). In a recent sister species comparison, both carnitine and acetyl carnitine were more abundant in DT *S. lepidophylla* than in DS *S. moellendorffii* (Yobi et al., 2012). However, in the current study, carnitine was not detected and acetyl carnitine was more abundant in the hydrated state, except in DRY-1 (Supplemental Table 1).

Ophthalmate (glu-2-aminobutyrate-gly), a glutathione pathway metabolite and analog of glutathione that lacks antioxidant properties, was more abundant in all dry or partially dry states compared with the fully hydrated state, and significantly so in DRY-1 (Supplemental Table 1). This compound was only recently reported in plants (Oliver et al., 2011). Its accumulation pattern resembles that observed in the DT grass *S. stapfianus*, in which ophthalmate abundance increased when RWC reached 50% or less; however, the functional significance of its accumulation remains unclear (Oliver et al., 2011).

Betaine (glycine betaine), a well-known osmoprotectant in many organisms including *E. coli* (Miller and Ingram, 2007) and plants (Chen and Murata, 2011), failed to display significant changes in abundance with changes in hydration states (Supplemental Table 1). However, this compound was more abundant in DT *S. lepidophylla* relative to DS *S. moellendorffii* (Yobi et al., 2012).

**Nucleotide Metabolism**

Aside from amino acids, the relative abundances of other nitrogen-rich metabolites within the purine and pyrimidine nucleotide pathways were altered during the rehydration/dehydration cycle in *S. lepidophylla*. Of the 14 nucleotides and derivatives detected, the relative amounts of three of them (e.g. allantoin, 1-methyladenosine, and uridine...
5’-monophosphate (UMP)) were significantly ($p < 0.05$) more abundant in all dry or partially dry states compared with the fully hydrated state, and significantly so in DRY-1 (Supplemental Table 1). Inosine was also more abundant in all of the dry or partially dry states, but this difference was not significant. In contrast, 2’-deoxyadenosine was significantly ($p < 0.05$) more abundant in fully hydrated S. lepidophylla compared with both dry and REH-50 states (Supplemental Table 1).

The ureides allantoin and allantoate serve as nitrogen-rich intermediates of purine catabolism that are involved in abiotic stress protection, apparently by quenching ROS (Werner and Witte, 2011). Both allantoin and allantoate can attenuate cell death and ROS accumulation in an Arabidopsis mutant defective in xanthine dehydrogenase, the key enzyme in the catabolism of purine leading to the production of hypoxanthine and xanthine and then to uric acid, which is subsequently degraded to allantoin and allantoate (Brychkova et al., 2008). Similarly, RNA interference-mediated suppression of xanthine dehydrogenase in drought-stressed Arabidopsis led to a marked reduction in plant growth and significant increases in cell death and H$_2$O$_2$ accumulation (Watanabe et al., 2010). However, this stress-hypersensitive phenotype can be reversed if the plants are pretreated with exogenous uric acid. These results demonstrate the importance of these ureides in nutrient recovery and remobilization during stress. The greater relative abundance of allantoin in all dehydrated or partially dehydrated states examined here points to similar roles for this compound in DT survival and recovery in S. lepidophylla. These results are also similar to those observed in the DT grass S. stapfianus, in which allantoin abundance increased when RWC fell to 40% or less (Oliver et al., 2011).

**Glutathione and γ-glutamyl Peptide Metabolism**

Within glutathione metabolism and the associated γ-glutamyl amino acid biosynthetic pathway, significant differences in the relative abundance of many metabolites were apparent over the course of the rehydration/dehydration cycle. For example, of the nine γ-glutamyl amino acid dipeptides detected, seven were more abundant in a dry state or partially dry state relative to the hydrated state, and four of these (e.g. γ-glutamylisoleucine, γ-glutamylleucine, γ-glutamylmethionine, and γ-glutamylthreonine) were significantly ($p < 0.05$) more abundant (Figure 7 and Supplemental Table 1). In contrast, γ-glutamyltryptophan was more abundant in the hydrated state, except in the DEH-50 state (Supplemental Table 1). Other pathway intermediates, including 5-oxoproline, were up to fivefold more abundant in the hydrated state compared with the dry or partially dry states (Figure 7 and Supplemental Table 1). Glycine and oxidized glutathione (GSSG) were also significantly ($p < 0.05$) more abundant in the hydrated state, with the exception of the DEH-50 state (Figure 7 and Supplemental Table 1). As mentioned above, ophthalmate, a glutathione pathway metabolite, was more abundant in all dry or partially dry states than in the fully hydrated state (Supplemental Table 1).

In Arabidopsis, and presumably in other plants, γ-glutamyl amino acid cycle γ-glutamyltranspeptidase (also known as γ-glutamyltransferase) catalyzes the interconversion of

![Figure 7](image_url)
glutathione (GSH, L–Glu–Cys–Gly) and free amino acids to cysteinylglycine and γ-glutamyl amino acids (Ohkama-Ohtsu et al., 2008). In animal systems, γ-glutamyl cyclo-transferase converts γ-glutamyl amino acids into 5-oxoproline, releasing the amino acid previously captured in the extracellular space into the cytoplasm (Ohkama-Ohtsu et al., 2008). In Arabidopsis, GSH degradation occurs within the cytoplasm and transmembrane recycling of amino acids is thought to be a minor event (Ohkama-Ohtsu et al., 2008). In S. lepidophylla, the significant increases in γ-glutamyl amino acids, as well as nitrogen-rich amino acids such as glutamate, during all stages of dehydration suggest that these peptides might have an important function in the acquisition of DT. For example, transmembrane amino acid recycling might occur as it does in animal systems, and thus provide an important mechanism for nitrogen storage during desiccation, as suggested to occur in S. stapfianus (Oliver et al., 2011). Moreover, the accumulation of γ-glutamyl amino acids in response to desiccation appears to be an evolutionarily well-conserved event. For example, γ-glutamylisoleucine, γ-glutamylleucine, and γ-glutamyl-phenylalanine accumulate in S. lepidophylla (Figure 7), as well as in T. ruralis and S. stapfianus, following desiccation (Oliver et al., 2011). In addition, both S. lepidophylla and S. stapfianus accumulate γ-glutamylglutamine and γ-glutamylmethionine, whereas both S. lepidophylla and T. ruralis accumulate γ-glutamylvaline (Oliver et al., 2011). In addition, S. lepidophylla accumulates γ-glutamylthreonine, but not in a significant manner (Supplemental Table 1). Sister-group comparisons between DS and DT species of Sporobolus (Oliver et al., 2011) and Selaginella (Yobi et al., 2012) demonstrated that DS species fail to accumulate γ-glutamyl amino acids, providing further support for an important role for these compounds in DT. Although additional research is needed on these compounds, storage of γ-glutamyl amino acids in the dried state could provide a readily mobilized form of nitrogen for protein synthesis following rehydration.

GSH exists in either an oxidized or a reduced form. The oxidized form of glutathione (GSSG) showed significantly (p < 0.05) greater abundance in S. lepidophylla in the hydrated or partially hydrated states (Figure 7). Upon becoming oxidized, it donates a reducing equivalent to unstable reactive oxygen intermediates such as hydrogen peroxide or dehydroascorbate, and then reacts instantly with a similar molecule to form glutathione disulfide (GSSG) (Mittler, 2002). Therefore, the greater abundance of GSSG observed in hydrated S. lepidophylla suggests active protection against oxidative stress at early stages of dehydration. In contrast, S. stapfianus did not show significant accumulation of GSSG during DT until RWC reached <40% (Oliver et al., 2011).

In terms of other antioxidant systems, S. lepidophylla failed to display the desiccation-induced accumulation of alpha- and beta-tocopherols as was observed in S. stapfianus (Oliver et al., 2011). This observation suggests that ROS scavenging occurs in a constitutive manner in S. lepidophylla, which is consistent with the constitutive production of several other classes of metabolites including sugars and sugar alcohols discussed earlier.

Secondary Metabolites
There were no significant differences in the relative abundance of secondary metabolites over the course of the rehydration/dehydration cycle with the exception of vanillate, which was more abundant in the hydrated states and significantly so compared with the DRY-2 state (Supplemental Table 1). Vanillate (and vanillic acid esters) is a well-known and potent antioxidant that exhibits protective effects against free radical-induced biomembrane damage (Tai et al., 2012). Vanillate appears to be important in DT, as this compound was found to be significantly more abundant in both sister-group comparisons between DS and DT species of Sporobolus (Oliver et al., 2011) and Selaginella (Yobi et al., 2012).

In contrast, other phenolics (e.g. caffeate), flavonols (e.g. apigenin and naringenin), and phenylpropanoids (e.g. coniferyl alcohol) were more abundant in all dry or partially dry states, but these differences were not significant (Supplemental Table 1). A flavonoid closely related to apigenin, called saponarin, is known to be induced in barley seedlings upon 6–8 d of UV-B exposure (Kaspar et al., 2010). Thus, apigenin is very likely to play a protective role against irradiation with UV light. In addition, apigenin likely ameliorates oxidative stress, as shown in mammalian cells (Chan et al., 2012; Suh et al., 2012). Naringenin, which is closely related in structure to apigenin, is also likely to play protective roles against both exposure to UV light and oxidative stress damage, as demonstrated in rat kidney (Renugadevi and Prabu, 2009) and hepatic (Prabu et al., 2011) cells. Apigenin, naringenin, and vanillate showed significantly (p < 0.05) greater abundance in DT S. lepidophylla compared with DS S. moellendorffii at two different hydration states (Yobi et al., 2012), reinforcing the importance of these compounds in DT.

Lipids, Phospholipids, and Fatty Acids
Of the 32 lipid metabolism compounds surveyed, only one (i.e. choline phosphate) showed greater relative abundance in response to dehydration at all stages of dehydration and significantly (p < 0.05) so in both dry states, whereas 12 compounds were significantly more abundant in the fully hydrated state compared with one or more re- or dehydration states (Supplemental Table 1). The zwitterionic choline phosphate head group is known to possess a strong water-association capacity and appears to promote water-lipid association between the onium head group and O=C=O groups in fatty acid chains (Foglia et al., 2010). Water molecules bound at this interphase might promote interaction with biomolecules such as sugars, amino acids, and membrane proteins (Disalvo et al., 2008). Membrane interaction with simple sugars or oligosaccharides has been postulated to stabilize or protect the native
structure of lipid bilayers through inhibition of the fluid-to-gel membrane phase transition at low hydration (Ohtake et al., 2006; Valluru and Van den Ende, 2008). Thus, the increased relative abundance of choline phosphate might promote membrane hydration as well as aid in membrane stabilization during the various stages of the rehydration/dehydration cycle.

Several lipoxygenase (LOX) activity markers (e.g. 13-hydroxy octadecadienoic acid (13-HODE) and 2-hydroxypalmitate) showed greater relative abundance under a majority of dry or partially dry states, but these differences were not significant (Supplemental Table 1). These lipid peroxidation products might arise during dehydration from either ROS attack or the action of LOXs (Marin et al., 1998). In addition, compounds such as 13-HODE, 2-hydroxypalmitate, and 2-hydroxystearate exhibited significantly (p < 0.05) greater abundance in DT S. lepidophylla compared with DS S. moellendorffii at two different hydration states, suggesting these compounds participate in DT mechanisms (Yobi et al., 2012). Several unsaturated fatty acids (e.g. arachidate, dihomo-linoleate, dihomo-linolenate, linoleate, and linolenate) were significantly (p < 0.05) more abundant in the hydrated state compared with the DRY-1 or DRY-2 states (Supplemental Table 1). A decrease in the relative amounts of various unsaturated lipids upon dehydration has been documented in the DT angiosperms S. stapfianus (Quartacci et al., 1997) and Ramonda serbica (Quartacci et al., 2002). These alterations in unsaturated fatty acid concentrations might contribute to maintaining or increasing membrane fluidity to allow for recovery following dehydration (Upchurch, 2008). Thus, the observed trend towards greater unsaturated fatty acid abundance in the hydrated state and during dehydration suggests a predisposition towards the maintenance of membrane fluidity in S. lepidophylla.

Unnamed Metabolites

Of the 84 unnamed compounds identified in S. lepidophylla, 15 (17.8%) and 17 (20.2%) were significantly (p < 0.05) more abundant in one or more dry or partially dry states than the fully hydrated state, respectively. Unnamed compounds significantly more abundant in dry or partially dry states showed 2.1–7.3-fold greater abundance, respectively. The unnamed compounds significantly more abundant in the fully hydrated state displayed 2.0–4.0-fold greater abundance compared to dry or partially dry states, respectively. In addition, nine and five unnamed compounds were always more abundant in all dehydrated states and in the hydrated state, respectively. However, these hydration-state-specific (dry/partially dry or fully hydrated) differences were not significant (Supplemental Table 1).

The relatively small magnitude of significant differences among the five different hydration states in S. lepidophylla suggests that metabolites that might play key roles in DT are mainly expressed constitutively. This idea is reinforced by the observation that no unnamed compound showed more than a 1.7-fold increase in relative abundance in the DEH-50 state (Supplemental Table 1). This observation stands in stark contrast to the 14 unnamed metabolites that showed 5–120-fold greater abundance in S. lepidophylla from a sister-group contrast between S. lepidophylla and S. moellendorffii at 50% and 100% RWC hydration states (Yobi et al., 2012). Nonetheless, the seven unnamed metabolites that showed >2.0-fold greater abundance under dry or partially dry conditions in S. lepidophylla represent important targets for future research, as these compounds might play important roles in the acquisition of DT and could also serve as useful targets for metabolic engineering to improve drought tolerance in crops. Lastly, 11 unnamed compounds showed significantly increased abundance in the REH50 state (Figure 8), which suggests that these compounds might play roles during the recovery from desiccation. Because S. lepidophylla is positioned evolutionarily between bryophytes, which rely mostly upon repair-based mechanisms during rehydration, and angiosperms, which rely mainly upon dehydration-induced protection (Oliver et al., 2000a), S. lepidophylla provides a useful model to bridge our understanding of the evolutionary progression of DT mechanisms among distantly related resurrection species.

Conclusions

This temporal evaluation of a five-stage, rehydration/dehydration cycle in S. lepidophylla afforded a global, non-biased, high-resolution account of the metabolite abundance changes that occur as part of the ability of this spike moss to ‘resurrect’ from an air-dried state. Like DT mosses, S. lepidophylla exhibits an obvious predisposition for DT by expressing a majority of metabolites, such as highly abundant trehalose, sucrose, and glucose in a constitutive manner, whereas only 16.7% and 29.5% of all compounds show significant increases or decreases in abundance upon change in hydration state, respectively. Many glycolysis/gluconeogenesis and TCA cycle intermediates and many different sugar alcohols and sugar acids were more abundant in the hydrated state. Polysols appear to play various roles, including the slowing of water loss during drying or water absorption during rehydration. Polysols might also serve as osmoprotectants in stabilizing or preventing denaturation of proteins during dehydration, in hydroxyl radical scavenging, in redox control, in reducing ROS production, and in triggering stress-adaptive gene expression. Vanillate was more abundant in the hydrated states and is a well-known and potent antioxidant that is likely to protect against free radical-induced biomembrane damage. Protection against photo- and oxidative damage during dehydration is also known to be critical for DT. Citrulline, a non-protein amino acid analog of arginine, and allantoin, a nitrogen-rich product of purine catabolism, which likely serve as nitrogen reserves or attenuate ROS accumulation and cell death, were more abundant in all dry and partially dry states. Several amino acid-derived secondary metabolites also appeared to play protective roles against UV-light damage including 3-(3-hydroxyphenyl) proponate and the flavonols apigenin and naringenin in all dry...
and partially dry states. In addition, several nitrogen-rich and γ-glutamyl amino acids that were markedly more abundant in dry or partially dry *S. lepidophylla*, likely play critical roles in the acquisition of DT through the scavenging of ROS via glutathione metabolism, or the remobilization of amino acids following rehydration, or both. Lastly, *S. lepidophylla* contained seven unnamed compounds that displayed twofold or greater abundance in dry or rehydrating states, suggesting that these compounds might play adaptive roles in the acquisition of DT or in the recovery from the desiccated state.

**METHODS**

**Plant Material and Water-Deficit Stress Treatments**

*Selaginella lepidophylla* (Hooker and Greville) Spring (flower of stone) plants were purchased in the dried state from Hirt’s Gardens (Medina, OH) and kept dry until the plants were subjected to an initial rehydration/dehydration cycle in order to identify and remove nonviable tissues. The rate of RWC change during the rehydration/dehydration cycle was established by submerging nine approximately 1-year-old plants in distilled water in a growth chamber under constant (175 μmol m⁻² s⁻¹) cool-white fluorescent and incandescent light at 26°C and 37% relative humidity (RH). For hydration, the plants were removed from the water, excess water was removed by gentle blotting, and the plants were weighed at regular intervals over a 24-h period. For dehydration, the plants were then placed in dry trays and weighed at regular intervals over a 24-h period. The plants were incubated at 65°C for 2 d, then reweighed to obtain dry weights. The RWC was calculated using the following formula: \[ \text{RWC (\%) = } \frac{(\text{Fwt-Dwt})}{(\text{Ftwt-Dwt})} \times 100, \] where Fwt is the fresh weight at specific time points during the rehydration/dehydration cycle.

![Figure 8](image-url)  
*Figure 8.* Differences in Nine Unnamed Compounds in *S. lepidophylla* Profiled during a Rehydration/Dehydration Cycle. Compounds with greater relative abundance in one or more dry states as shown in box plots are presented. Box plots are as described in the Figure 4 legend. The *p*- and *q*-values for the comparisons are presented in Supplemental Table 1.
Dwt is the weight after incubation at 65°C for 2 d, and FTwt is the weight after 24 h of rehydration. Samples representing five RWCs—the initial dry state (DRY-1), 50% rehydrated (REH-50), 100% rehydrated (HYD), 50% dehydrated (DEH-50), and completely dehydrated (DRY-2)—were then selected to determine differences in metabolite composition over the course of the rehydration/dehydration cycle.

Metabolomic Profiling

Six biological replicates from each species were collected at each time point, lyophilized, and kept at −80°C under hermetic conditions prior to extraction. Then, 20 mg of each leaf sample was extracted in 400 μl of methanol containing recovery standards using an automated MicroLab STAR® system (Hamilton Company, Salt Lake City, UT).

Global unbiased metabolite profiling in *S. lepidophylla* was performed using an integrated platform consisting of a combination of three independent approaches: (1) ultra-high performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS²) optimized for basic species, (2) UHLC/MS/MS² optimized for acidic species, and (3) gas chromatography/mass spectrometry (GC/MS) as described in detail in Evans et al. (2009).

UHLC/MS/MS² analyses were performed using a Waters Acquity UPLC (Waters Corporation, Milford, MA) and a Thermo-Finnigan LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) equipped with an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. Each sample was subjected to two independent UHPLC/MS runs using separate dedicated columns optimized for either positive ions or for negative ions. For GC/MS, samples were re-dried under vacuum desiccation for a minimum of 24 h, derivatized under dried nitrogen using bistrimethylsilyl-trifluoroacetamide (BSTFA), and then analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS using electron impact ionization operated at unit mass resolving power. Chromatographic separation, followed by full scan mass spectra, was carried out to record retention time, molecular weight (m/z), and MS/MS² of all detectable ions present in the samples (see Supplemental Table 2) in compliance with recommendations for reporting metabolite data (Fernie et al., 2011).

Automated comparison of the ion features in the experimental samples was used to identify metabolites using a custom reference library of chemical standards. Reference library standard entries included the retention time, molecular weight (m/z), preferred adducts, and in-source fragments of compounds, as well as their associated MS² spectra. Comparison of experimental samples to process blanks (water only) and solvent blanks allowed for the removal of artifactual peaks, whereas the reference library allowed the rapid identification of metabolites in the experimental samples with high confidence. Mapping of named metabolites was performed onto general biochemical pathways, as provided in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg) and Plant Metabolic Network (PMN) (www.plantcyc.org).

Data Imputation and Statistical Analysis

The samples were analyzed over the course of 2 d. This required data correction for minor variations resulting from instrument inter-day tuning differences (Evans et al., 2009). If values for a given metabolite were missing, then these were assigned the observed minimum detection value, based on the assumption that the missing values were below the limits of detection. For the convenience of data visualization, the raw area counts for each biochemical were rescaled by dividing each sample value by the median value for the specific biochemical.

Statistical analysis of the data was performed using JMP (SAS, www.jmp.com), a commercial software package, and ‘R’ (http://cran.r-project.org/), a freely available open-source software package. A log transformation was applied to the observed relative abundances for each biochemical because the variance generally increased as a function of each biochemical’s average response.

Out of the 251 metabolites that were detected, a total of 204 metabolites with no missing values were imported into the chemometrics software Solo (Eigenvector Research, Inc., Wenatchee, WA) for Partial Least Squares-Discriminant Analysis (PLS-DA) to determine classification of the different treatments (Figure 3). PLS-DA is a regression extension of Principal Component Analysis (PCA) used to model group classification. *R*² and *Q*² are used as measures for the robustness of a PLS-DA model, where *R*² is the cumulative variance explained by the components. Cross-validation of *R*² estimates *Q*², which is the cumulative variance predicted by the model. Thus, both of these values indicate how well the overall model classifies and predicts group membership in a data set. Both of these values range from 0 to 1 and the higher the number, the more robust the model.

In order to visualize the entire data set, a heat map was generated to show fold change for each compound identified from the LC-MS/MS² or GC-MS analyses of the tissue samples (see Supplemental Table 1). Fold change was calculated for each compound defined as the means ratio of each treatment in *S. lepidophylla*. Welch’s two-sample *t*-tests were then used to determine whether or not each metabolite had significantly increased or decreased in abundance. The False Discovery Rate (FDR) was then calculated to correct for multiple Welch’s two-sample *t*-test comparisons for the hundreds of compounds detected. Metabolite expression studies are very similar to gene array studies with a very large number of statistical comparisons. As with microarray studies, metabolomic profiling generates large numbers of metabolites, and thus FDR is typically calculated with multiple comparison adjustment instead of family-wise error rate adjustments, such as the Bonferroni or Tukey corrections. The FDR for a given set of metabolites...
was estimated by the q-value (Storey, 2002). Box plots were generated for those compounds that showed a significant increase or decrease using both the Welch two-sample t-test and FDR (i.e. \( p < 0.05 \) and \( q < 0.10 \)) significance values.

**SUPPLEMENTARY DATA**

Supplementary Data are available at Molecular Plant Online.

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