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## **EVOLUTION OF SALT TOLERANCE IN ANGIOSPERMS: A PHYLOGENETIC APPROACH**

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### **ABSTRACT**

Salt tolerance in plants involves a number of physiological, anatomical, and morphological traits that mitigate the effects of osmotic and ionic stress of salinity. Despite the complexity of adaptations to environmental salinity, salt tolerance has arisen independently in many different lineages of flowering plants. In this chapter, we discuss phylogenetic perspectives on the study of salt tolerance. Although few angiosperm species are halophytes, salt tolerance appears to be evolutionarily labile, with relatively numerous instances of independent evolutionary origins. Salt tolerance evolves more often in some lineages than others, which may reflect the fact that it builds upon enabling pre-existing traits in those lineages that facilitate the evolution of tolerance to a wider range of environmental stresses. We describe phylogenetic comparative analyses that explore the evolutionary association between salt tolerance and other related ecophysiological strategies: C<sub>4</sub> photosynthesis, heavy metal tolerance, and alkali-tolerance. However, we discuss the limitations in identifying causality and direction of causality of these associations. We conclude that phylogenetic comparative studies can help us to understand the evolutionary dynamics of salt tolerance in angiosperms and potentially aid identification of shared mechanisms underlying tolerance to several environmental stresses. Despite outstanding challenges in conducting these studies, these comparative approaches have the potential to reveal evolutionary associations between different tolerances to environmental stress.

## INTRODUCTION

High levels of environmental salt can occur in most biogeographic regions both at land and sea, inland and at the coast, across a range of ecological settings, such as marshes, mangroves, deserts, salt lakes, and the ocean (Reimond & Queen, 1974). These areas of high salinity are often inhabited by plants that survive despite the twofold difficulties that salt poses to plant functions. Firstly, high salinity decreases plants' ability to take up water. Secondly, high concentrations of  $\text{Na}^+$  and  $\text{Cl}^-$  within the plant impair metabolic processes and decrease photosynthetic efficiency, which in turn negatively affect plant growth (Flowers & Yeo, 1995; Mäser et al., 2002). Salt tolerance refers to various morphological, physiological, and biochemical adaptations that enable plants to survive and complete their life cycles in saline environments. Such plant species are often referred to as halophytes. Salt tolerance adaptations deal with both the osmotic stress, as well as the ionic stress caused by  $\text{Na}^+$  (Munns & Tester, 2008; Sahi et al., 2006) and  $\text{Cl}^-$  (Teakle & Tyerman, 2010). The effects of osmotic stress are reduced by decreasing water loss while maximising water uptake and overall water use efficiency through reduced cellular expansion rates and tight packing of photosynthetic proteins (Munns & Tester, 2008; Reich et al., 1999; Wright et al., 2003). Ion toxicity is mitigated by maintaining a favourable osmotic gradient either through the compartmentalisation of toxic ions in specialised vacuoles and the production of compatible organic solutes (Blumwald, 2000; Glenn et al., 1999; Munns & Tester, 2008), or with the secretion of salt via specialised glands (Munns & Tester, 2008).

Although flowering plants are found in saline environments across the globe, halophytes are comparatively rare. In fact, only an estimated 0.25% of all flowering plant species are known to be halophytes (Flowers et al., 2010). However, halophytes are an extremely diverse group of species. They vary in their degree of salt tolerance, which they achieve through different traits (Flowers & Colmer, 2008), as well as in their life forms, which include annual and perennial herbs, shrubs, and trees (Rozema & Flowers, 2008). Furthermore, halophytes come from a wide range of angiosperm lineages, suggesting that the adaptations involved in salt tolerance have arisen repeatedly during angiosperm evolution (Flowers et al., 1977; Flowers et al., 2010).

Studies of the evolution of salt tolerance can address a series of evolutionary questions. Given that salt tolerance is a complex trait, how frequently has it evolved in angiosperms? How can we unveil the mechanisms underlying this evolutionary transition from glycophytes to halophytes? What conditions have facilitated this transition? This chapter explores what we can infer about evolutionary patterns of salt tolerance from phylogenetic analyses. We compare evolutionary patterns of salt tolerance with other adaptations to environmental stresses, such as modified photosynthetic routes, heavy metals and alkalinity, and discuss how these traits could be associated.

## A PHYLOGENETIC VIEW OF SALT TOLERANCE IN ANGIOSPERMS

Analysing the phylogenetic distribution of halophytes is a key step in understanding the evolution of salt tolerance in angiosperms. This topic has been explored at the broad scale (Flowers et al., 1977), and we know that halophytes are found in at least 20 diverse

angiosperm orders. Given the relationship between these orders, it is likely that salt tolerance has originated multiple times independently over the evolutionary history of angiosperms (Flowers et al., 2010). Within some groups like the chenopods (Kadereit et al., 2012) and sea grasses (Les et al., 1997), salt tolerance seems to have been gained early in the evolutionary history of those groups and lost rarely, if at all. However, a recent study inferred at least 70 independent origins of salt tolerance in the grass family (Bennett et al., 2013). Most reconstructed origins were near the tips of the phylogeny, and gave rise to few halophyte species (Bennett et al., 2013). This finding suggests that, although salt tolerance is a complex trait, it has evolved multiple times. The difference in the phylogenetic pattern of distribution of halophytes among different families shows that salt tolerance can have different evolutionary patterns in different lineages, which warrants further investigation.

### **Taxonomic Distribution of Halophytes**

In this chapter, we explore the distribution of known halophytes across angiosperm families to investigate if halophytes are distributed randomly across angiosperms. We first recorded the angiosperm families recognised by the APG III and the number of species estimated in each family (<http://www.mobot.org/MOBOT/research/APweb/>). We then found the number of known halophyte species in each angiosperm family recognised by the APG III, based on a published list of halophytes (Menzel & Lieth, 2003). This list provides approximately 2,600 names of plant species reported as halophytes in published studies based on ecological, physiological and anecdotal data (Menzel & Lieth, 2003). Although no published list of halophytes will be complete, due to poor knowledge of salt tolerance in certain families and geographical regions, we believe this is the most extensive published database of known halophytes.

We found the accepted name of each halophytic species in that list by searching The Plant List (2010) with the package “taxonstand” (Cayuela et al., 2012) in the program R (R Core Team, 2014). We then allocated each accepted halophyte species to its respective family using the taxonomic name resolution service [TNRS (Boyle et al., 2013)]. Using this method of estimation, we identified 1,653 halophytic species (Table 1). Based on this survey, we found that halophytes are distributed in 117 families and 34 orders. As expected based on previous studies (Flowers et al., 1977; Flowers et al., 2010), many of the families with the highest proportions of halophytes (Table 1) come from the orders Alismatales (including sea grasses) and Caryophyllales (including chenopods). However, there are several families with relatively high proportions of halophytes within the orders Malphigiales, Fagales, and Zygophyllales.

### **Phylogenetic Distribution of Halophytes**

The distribution of halophytes among taxonomic groups shows that halophytes are found in at least a quarter of angiosperm families. However, we cannot assume that the 117 families with halophytes evolved salt tolerance independently. To estimate the number of origins of salt tolerance across angiosperm families, we carried out a phylogenetic investigation.

**Table 1. Estimates for number and percentage of halophytes for 117 families recognised by APG III containing at least one known halophyte. Family names, orders, and estimated species numbers were taken from the APG website version 13 (<http://www.mobot.org/MOBOT/research/APweb/>). Number of halophytes was derived from the set of accepted halophyte species included in Haloph v2 (Menzel & Lieth, 2003) based on The Plant List (2010), and their respective family affinities according to the Taxonomic Name Resolution Service (Boyle et al., 2013). We highlight families with more than 50 species in bold. Families are ranked alphabetically by the order to which they belong**

Order	Family	Number of Species	Number of Halophytes	Percentage of Halophytes
Alismatales	<b>Alismataceae</b>	88	1	<b>1.14</b>
Alismatales	Cymodoceaceae	16	12	75.00
Alismatales	<b>Hydrocharitaceae</b>	116	13	<b>11.21</b>
Alismatales	Juncaginaceae	15	1	6.67
Alismatales	Posidoniaceae	9	3	33.33
Alismatales	<b>Potamogetonaceae</b>	102	7	<b>6.86</b>
Alismatales	Ruppiceae	6	1	16.67
Alismatales	Zosteraceae	14	14	100.00
Apiales	<b>Apiaceae</b>	3780	9	<b>0.24</b>
Apiales	<b>Araliaceae</b>	1450	3	<b>0.21</b>
Arecales	<b>Arecaceae</b>	2361	29	<b>1.23</b>
Asparagales	<b>Amaryllidaceae</b>	1605	4	<b>0.25</b>
Asparagales	<b>Asparagaceae</b>	2480	9	<b>0.36</b>
Asparagales	<b>Iridaceae</b>	2025	4	<b>0.20</b>
Asparagales	<b>Xanthorrhoeaceae</b>	900	1	<b>0.11</b>
Asterales	<b>Asteraceae</b>	23600	117	<b>0.50</b>
Asterales	<b>Calyceraceae</b>	60	2	<b>3.33</b>
Asterales	<b>Campanulaceae</b>	2380	1	<b>0.04</b>
Asterales	<b>Goodeniaceae</b>	430	4	<b>0.93</b>
Brassicales	Bataceae	2	2	100.00
Brassicales	<b>Brassicaceae</b>	3710	21	<b>0.57</b>
Brassicales	<b>Capparaceae</b>	480	1	<b>0.21</b>
Brassicales	<b>Cleomaceae</b>	300	3	<b>1.00</b>
Brassicales	<b>Resedaceae</b>	75	1	<b>1.33</b>

Order	Family	Number of Species	Number of Halophytes	Percentage of Halophytes
Brassicales	Salvadoraceae	11	1	9.09
Caryophyllales	<b>Aizoaceae</b>	2035	36	<b>1.77</b>
Caryophyllales	<b>Amaranthaceae</b>	2275	393	<b>17.27</b>
Caryophyllales	Anacampserotaceae	32	1	3.13
Caryophyllales	Basellaceae	19	2	10.53
Caryophyllales	<b>Cactaceae</b>	1866	8	<b>0.43</b>
Caryophyllales	<b>Caryophyllaceae</b>	2200	17	<b>0.77</b>
Caryophyllales	Didiereaceae	16	2	12.50
Caryophyllales	<b>Frankeniaceae</b>	90	15	<b>16.67</b>
Caryophyllales	Halophytaceae	1	1	100.00
Caryophyllales	<b>Molluginaceae</b>	87	1	<b>1.15</b>
Caryophyllales	<b>Montiaceae</b>	226	3	<b>1.33</b>
Caryophyllales	<b>Nyctaginaceae</b>	395	7	<b>1.77</b>
Caryophyllales	<b>Plumbaginaceae</b>	836	28	<b>3.35</b>
Caryophyllales	<b>Polygonaceae</b>	1110	22	<b>1.98</b>
Caryophyllales	<b>Portulacaceae</b>	70	5	<b>7.14</b>
Caryophyllales	Sarcobataceae	2	1	50.00
Caryophyllales	Stegnospermataceae	3	1	33.33
Caryophyllales	Talinaceae	27	2	7.41
Caryophyllales	<b>Tamaricaceae</b>	90	28	<b>31.11</b>
Celastrales	<b>Celastraceae</b>	1400	8	<b>0.57</b>
Commelinales	Pontederiaceae	33	1	3.03
Cucurbitales	<b>Cucurbitaceae</b>	960	3	<b>0.31</b>
Dilleniales	<b>Dilleniaceae</b>	355	1	<b>0.28</b>
Ericales	<b>Ebenaceae</b>	548	2	<b>0.36</b>
Ericales	<b>Ericaceae</b>	3995	1	<b>0.03</b>
Ericales	<b>Lecythidaceae</b>	310	4	<b>1.29</b>
Ericales	<b>Primulaceae</b>	2590	10	<b>0.39</b>
Ericales	<b>Sapotaceae</b>	1100	2	<b>0.18</b>
Ericales	Tetrameristaceae	5	1	20.00
Fabales	<b>Fabaceae</b>	19500	113	<b>0.58</b>

**Table 1. (Continued)**

Order	Family	Number of Species	Number of Halophytes	Percentage of Halophytes
Fabales	Surianaceae	8	1	12.50
Fagales	<b>Casuarinaceae</b>	95	9	<b>9.47</b>
Gentianales	<b>Apocynaceae</b>	4555	20	<b>0.44</b>
Gentianales	<b>Gentianaceae</b>	1655	4	<b>0.24</b>
Gentianales	<b>Rubiaceae</b>	13150	4	<b>0.03</b>
Lamiales	<b>Acanthaceae</b>	4000	13	<b>0.33</b>
Lamiales	<b>Bignoniaceae</b>	800	8	<b>1.00</b>
Lamiales	<b>Lamiaceae</b>	7173	5	<b>0.07</b>
Lamiales	<b>Orobanchaceae</b>	2060	12	<b>0.58</b>
Lamiales	<b>Phrymaceae</b>	188	3	<b>1.60</b>
Lamiales	<b>Plantaginaceae</b>	1900	10	<b>0.53</b>
Lamiales	<b>Scrophulariaceae</b>	1800	10	<b>0.56</b>
Lamiales	<b>Verbenaceae</b>	918	12	<b>1.31</b>
Lamiales	<b>Boraginaceae</b>	2755	14	<b>0.51</b>
Laurales	<b>Lauraceae</b>	2500	2	<b>0.08</b>
Magnoliales	<b>Annonaceae</b>	2220	1	<b>0.05</b>
Malpighiales	<b>Calophyllaceae</b>	460	1	<b>0.22</b>
Malpighiales	<b>Chrysobalanaceae</b>	460	1	<b>0.22</b>
Malpighiales	<b>Clusiaceae</b>	595	1	<b>0.17</b>
Malpighiales	Elatinaceae	35	2	5.71
Malpighiales	<b>Euphorbiaceae</b>	5735	17	<b>0.30</b>
Malpighiales	<b>Hypericaceae</b>	560	1	<b>0.18</b>
Malpighiales	<b>Linaceae</b>	300	1	<b>0.33</b>
Malpighiales	<b>Phyllanthaceae</b>	1745	2	<b>0.11</b>
Malpighiales	<b>Putranjivaceae</b>	210	1	<b>0.48</b>
Malpighiales	<b>Rhizophoraceae</b>	149	19	<b>12.75</b>
Malpighiales	<b>Salicaceae</b>	1010	3	<b>0.30</b>
Malvales	<b>Malvaceae</b>	4225	27	<b>0.64</b>
Malvales	<b>Thymelaeaceae</b>	891	2	<b>0.22</b>
Myrtales	<b>Combretaceae</b>	500	10	<b>2.00</b>

Order	Family	Number of Species	Number of Halophytes	Percentage of Halophytes
Myrtales	<b>Lythraceae</b>	620	9	<b>1.45</b>
Myrtales	<b>Myrtaceae</b>	4620	22	<b>0.48</b>
Myrtales	<b>Onagraceae</b>	656	1	<b>0.15</b>
Nymphaeales	<b>Nymphaeaceae</b>	58	1	<b>1.72</b>
Pandanales	<b>Pandanaceae</b>	885	10	<b>1.13</b>
Picramniales	Picramniaceae	49	1	2.04
Piperales	<b>Piperaceae</b>	3615	1	<b>0.03</b>
Piperales	Saururaceae	6	1	16.67
Poales	<b>Bromeliaceae</b>	1770	2	<b>0.11</b>
Poales	<b>Cyperaceae</b>	5430	70	<b>1.29</b>
Poales	Flagellariaceae	4	1	25.00
Poales	<b>Juncaceae</b>	430	14	<b>3.26</b>
Poales	<b>Poaceae</b>	11160	212	<b>1.90</b>
Poales	<b>Restionaceae</b>	500	1	<b>0.20</b>
Poales	Typhaceae	25	6	24.00
Ranunculales	<b>Ranunculaceae</b>	2525	4	<b>0.16</b>
Rosales	Elaeagnaceae	45	2	4.44
Rosales	<b>Moraceae</b>	1125	5	<b>0.44</b>
Rosales	<b>Rhamnaceae</b>	925	5	<b>0.54</b>
Rosales	<b>Rosaceae</b>	2520	6	<b>0.24</b>
Rosales	Ulmaceae	35	1	2.86
Sapindales	<b>Anacardiaceae</b>	873	2	<b>0.23</b>
Sapindales	<b>Meliaceae</b>	615	3	<b>0.49</b>
Sapindales	Nitrariaceae	16	7	43.75
Sapindales	<b>Rutaceae</b>	2070	2	<b>0.10</b>
Sapindales	<b>Simaroubaceae</b>	110	1	<b>0.91</b>
Saxifragales	<b>Crassulaceae</b>	1370	1	<b>0.07</b>
Saxifragales	Cynomoriaceae	2	1	50.00
Solanales	<b>Convolvulaceae</b>	1625	14	<b>0.86</b>
Solanales	<b>Solanaceae</b>	2460	29	<b>1.18</b>
Vitales	<b>Vitaceae</b>	850	1	<b>0.12</b>
Zygophyllales	<b>Zygophyllaceae</b>	285	15	<b>5.26</b>

For this investigation we used the largest published tree of angiosperms, which contains over 56,000 angiosperm taxa and was constructed from publicly available sequences for six chloroplast and nuclear DNA markers (Smith et al., 2011). From this phylogenetic tree, we extracted a family-level phylogenetic tree, selecting one representative species for each family, randomly choosing between those species with the most sequence data in the alignment. We did not estimate branch lengths for this analysis, and used a phylogenetic tree with all branch lengths set to 1. We used the same list of halophytes described in the taxonomic analysis above, finding the accepted names of the species in a published list (Menzel & Lieth, 2003) according to The Plant List (2010), and using the TNRS (Boyle et al., 2013) to find family affinities. Using a parsimony ancestral state reconstruction method in Mesquite (Maddison & Maddison, 2006), we estimated that salt tolerance has evolved independently at least 59 times in the family-level phylogeny of angiosperms (Figure 1).

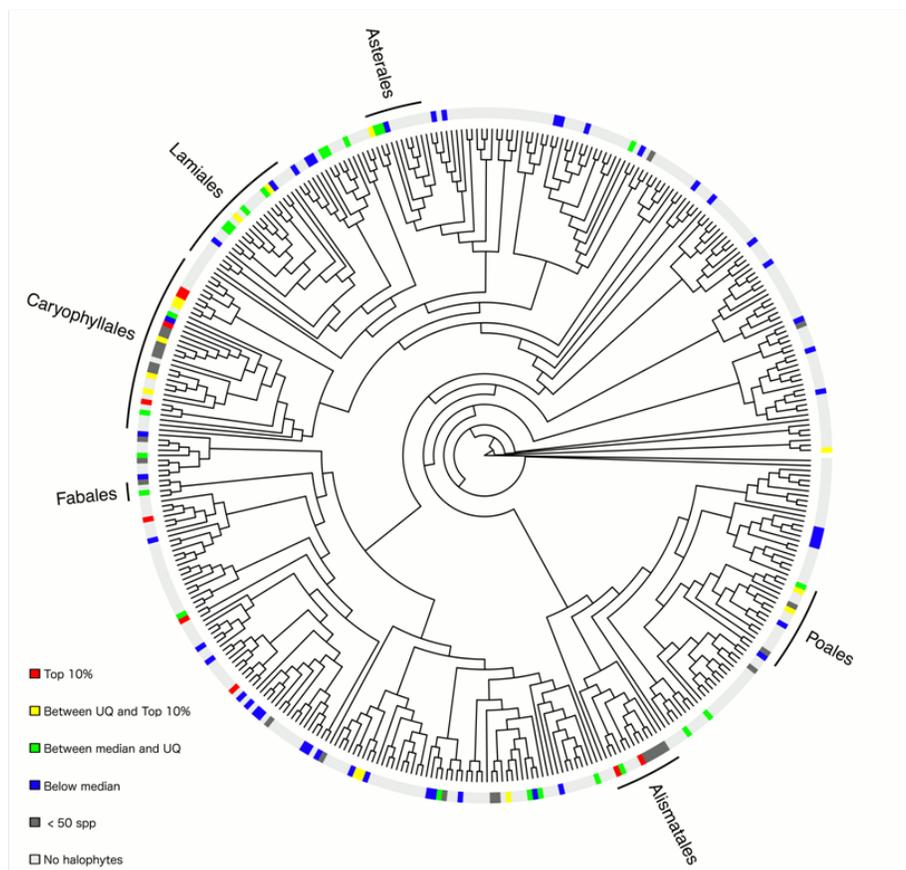


Figure 1. Phylogenetic tree of 401 APG III families extracted from a published angiosperm phylogeny (Smith et al., 2011). For each family, one representative taxon was selected based on maximum alignment length. Coloured tips represent families containing halophytes. Families were ranked by percentage of halophytes (see Table 1). Each tip is coloured based on the relative position of each family based on this ranking. We present families that were placed in top 10% of halophyte proportion (red), between the Upper Quartile (UQ) and the top 10% (yellow), between the median and the UQ (green), and below the median (blue). Families containing fewer than 50 species were not ranked and are shown in dark grey. Orders labelled on the phylogenetic tree contain at least 50 halophytes. The Figure was drawn with the R package “diversitree” (FitzJohn, 2012).

Although these origins are more prominent in some clades than others, they are dispersed on the phylogeny, with many close to the tips of the family-level tree, so are shared by only one or few families (Figure 1). Further, we explored the phylogenetic distribution of halophyte proportion within each family (Table 1). In Figure 1, we coloured the tips of the phylogeny according to halophyte proportion. We found that families with the highest proportion of halophytes do not appear to be clustered on the angiosperm family tree, but they are sometimes related to families with lower proportions of halophytes (Figure 1).

Of course, based on this result only, we cannot claim there have only been 59 origins of salt tolerance during the evolutionary history of angiosperms. Our analysis is at the family level and, although some families rarely lose salt tolerance [*e.g.*, chenopods (Kadereit et al., 2012), sea grasses (Les et al., 1997)], salt tolerance can be gained several times within a single family. For instance, in the Poaceae, which represent a single tip in our phylogenetic tree (Figure 1), we have identified over 70 origins of salt tolerance (Bennett et al., 2013). Therefore, if we expand our analysis to more shallow taxonomic levels, we expect that the number of estimated origins will only increase. However, it is not clear whether the labile evolutionary pattern of salt tolerance in the grasses is common across many families, or whether the factors driving salt tolerance evolution vary widely across lineages.

### **Future Phylogenetic Investigations of Salt Tolerance in Angiosperms**

The findings presented in this chapter highlight areas of research that require further investigation with regard to phylogenetic patterns of salt tolerance in angiosperms. First, future investigations can combine measures of ancestral state reconstruction, phylogenetic clustering and estimations of speciation and extinction to characterise the evolutionary processes underlying the origin of salt tolerance in different lineages.

Second, future investigations can explore the idea that evolutionary innovation, such as ecological transition to harsh environments, can be assisted by pre-existing enabling traits that make adaptation to these environments easier by providing starting points to environmental tolerance (Donoghue, 2005). Therefore, macroevolutionary investigations of the factors that have enabled the adaptive evolution of lineages to tolerate environmental stress can reveal mechanisms facilitating these evolutionary transitions (Edwards & Donoghue, 2013). For instance, the transition from C<sub>3</sub> to C<sub>4</sub> photosynthesis in grasses was facilitated by particular pre-existing foliar anatomy (large bundle sheath cells) in lineages that evolved C<sub>4</sub> photosynthesis (Christin et al., 2013). Similarly, a particular pre-existing three-dimensional leaf venation pattern in certain lineages has facilitated the evolution of succulence in those lineages, through the decoupling of leaf water storage from hydraulic path length (Ogburn & Edwards, 2013). Salt tolerance involves various traits, and not all of these traits are present in all halophytes. Therefore, it would be interesting to identify which of these “salt tolerance traits” have evolved in different salt-tolerant lineages. Can the evolution of different traits in different lineages, such as the presence of salt glands or the production of different compatible solutes, explain the contrasting evolutionary patterns of salt tolerance observed among lineages? In turn, these approaches could shed more light on the processes that allow complex traits to evolve repeatedly, particularly those involved in ecophysiological strategies as adaptations to harsh environments.

Third, it is worth exploring whether ascertainment bias in recording salt tolerance has an effect on the evolutionary patterns that we have observed so far. Salt tolerance is often recorded as a binary trait in evolutionary studies, species scored as being either salt tolerant or salt sensitive. However, salt tolerance can demonstrate considerable intraspecific variation, depending on physiological (Chapter 11; Cuartero et al., 1992; Lowry et al., 2009) and genetic factors (Nguyen et al., 2004; Nguyen et al., 2009; Rajakaruna et al., 2003). Tolerance to salinity would be better expressed on a continuum: not all halophytes can tolerate the same level of salinity and a lot of glycophytes are not completely salt-intolerant. For example, the study of the evolution of succulence in plants has suffered from the same bias as it has been treated as a binary trait (Edwards & Donoghue, 2013), but a quantitative measure of succulence has recently enabled more explicit tests of its evolution (Ogburn & Edwards, 2012). Similarly, exploring salt tolerance as a continuous variable on phylogenies might provide a more complete picture of the evolution of salt tolerance in angiosperms.

Finally, one question that can be explored is whether a better understanding of the evolution of salt tolerance can provide information for breeding salt tolerant crops. Salinity affects up to a tenth of Earth's land surface, and approximately half of all irrigated land around the globe (Ruan et al., 2010), with serious economic costs for agriculture. The majority of crop species do not grow well in saline conditions. Over the past few decades, important advances have been made in breeding salt tolerance into crops (Flowers, 2004; Rozema & Flowers, 2008). However, important challenges remain, in order to develop crop species that can successfully tolerate soil salinity. Because salt tolerance has already evolved in halophytes, it has been suggested that development of salt tolerant crops can be achieved through domestication of halophytes (Flowers, 2004), but many important limitations, such as variable germination and plant diseases of prospective crop halophytes and economic competition with conventional crops, have hindered this approach (Rozema & Flowers, 2008). Two ideas suggest that there could be practical applications to the evolutionary studies of salt tolerance for breeding salt tolerance. First, identifying close relatives of crop species with salt tolerance could help breed tolerance into crop species (Deinlein et al., 2014). Second, if we identify lineages that have more halophytes than expected, we could focus our search for new salt tolerant crops within these lineages, an approach that has been suggested for the search for medicinal plants (Saslis-Lagoudakis et al., 2012). Within those lineages, we could prioritise close relatives of known salt tolerant species for experimental screening of their tolerance (Joseph et al., 2013).

## **EVOLUTIONARY ASSOCIATIONS WITH OTHER STRESS-TOLERANCE TRAITS**

In this chapter so far, we have pointed out that salt tolerance has had multiple, recent independent origins across many angiosperm families. This evolutionary pattern reveals a paradox: salt tolerance is a rare and complex trait, but it is an evolutionarily labile one, which appears to be gained often. One possible explanation for the evolutionary lability of salt tolerance is that it builds upon a more general suite of stress-tolerance traits that enable lineages to adapt to a wide range of environmental challenges (Chapter 9; Chapin et al., 1993). This would mean that salt tolerance is more easily acquired in lineages with particular

background enabling traits as starting points (Bromham & Bennett, 2014), which might be linked to ecophysiological responses to aridity (Osborne & Freckleton, 2009; Rozema & Schat, 2013), and enhance the capacity of those lineages to evolve salt tolerance. One way to investigate this hypothesis is to employ a comparative phylogenetic approach (Christin et al., 2010; Hancock & Edwards, 2014). The strength of this approach is that it can place tolerance to several stresses within a unifying comparative framework. For any group of plants, we can plot the taxa with tolerance to different stresses on a phylogeny and then estimate the relatedness of taxa with resistance to these stresses. The detection of these associations on phylogenetic trees is the first step towards understanding their underlying causes. In the following paragraphs, we discuss the putative association of other types of stress tolerance with the evolution of salt tolerance. In particular, we focus on three ecophysiological traits: C<sub>4</sub> photosynthesis, heavy metal tolerance, and alkali-tolerance.

### **C<sub>4</sub> Photosynthesis**

C<sub>4</sub> photosynthesis is a modified photosynthetic pathway that increases a plant's efficiency of carbon fixation, thus reducing photorespiration and allowing higher water-use efficiency. In turn, C<sub>4</sub> ecophysiology conveys advantages in harsh environmental conditions, including salinity (Christin et al., 2013; Sage, 2004; Sage & Monson, 1999; Sage et al., 2012). In particular, the greater water-use efficiency in C<sub>4</sub> plants can not only reduce the effect of osmotic stress, but also mitigate the effects of ionic stress of salinity, because C<sub>4</sub> plants utilise less water, and therefore intake less salt, per growth unit (Sage, 2001). Many C<sub>4</sub> plants are known to be halophytes (Aronson, 1989; Dajic, 2006; Sage & Monson, 1999) and plants with C<sub>4</sub> photosynthesis are often overrepresented in saline environments (Eallonardo et al., 2013; Feldman et al., 2008). For example, plants with C<sub>4</sub> photosynthesis are dominant in saline Argentinian grasslands (Feldman et al., 2008). Like salt tolerance, C<sub>4</sub> photosynthesis is surprisingly evolutionary labile for a complex trait: C<sub>4</sub> photosynthesis has evolved independently over 60 times in angiosperms (Sage et al., 2012), including several times within the grass family (Edwards & Smith, 2010; Grass Phylogeny Working Group II, 2012). C<sub>4</sub> plant species are found scattered across the phylogeny of angiosperms (Edwards & Ogburn, 2012; Sage et al., 2011).

Previous studies have pointed out the putative association between C<sub>4</sub> photosynthesis and salt tolerance, as part of a wider stress syndrome to aridity (Edwards & Smith, 2010; Sage, 2001), and it has been suggested that the evolution of C<sub>4</sub> in grasses enabled the colonisation of arid and saline habitats (Osborne & Freckleton, 2009). Other studies have explicitly tested for this association in a phylogenetic context. For example, in the chenopods, higher rates of gain of C<sub>4</sub> were inferred in salt tolerant lineages, which was attributed to shared adaptations between C<sub>4</sub> photosynthesis and salt tolerance as part of a wider drought tolerance syndrome (Kadereit et al., 2012). Also, salt tolerance and C<sub>4</sub> photosynthesis are significantly associated in the grass family. Using a phylogeny of the grass family, and mapping halophytes and C<sub>4</sub> plants on that phylogeny, we have recently shown that salt tolerance is more likely to occur in C<sub>4</sub> than C<sub>3</sub> grass lineages (Bromham & Bennett, 2014).

However, although these studies have found a significant correlation on the phylogeny between C<sub>4</sub> photosynthesis and salt tolerance, it is not clear whether these traits are directly or indirectly linked. This association may suggest that some traits involved in C<sub>4</sub> photosynthesis

also directly convey tolerance to salinity or facilitate its evolution. On the other hand, it could be indirect factors that drive this association, such as biogeography.  $C_4$  photosynthesis facilitated the expansion of certain lineages into arid and saline habitats (Osborne & Freckleton, 2009; Strömberg, 2011), and this exposure may have indirectly rendered these lineages more likely to evolve salt tolerance. Further, even if these two traits have a direct causal connection, inferring the direction of this connection is challenging (Bromham & Bennett, 2014).

## **Heavy Metal Tolerance**

Another trait that has been suggested to be linked to salt tolerance is the ability to tolerate and accumulate heavy metals (Ghnaya et al., 2007; Manousaki & Kalogerakis, 2010; Rozema & Schat, 2013). Like salt, high concentrations of several heavy metals pose a lethal oxidative threat to most plants. However, some plant species—called heavy metal hyperaccumulators—are not only able to survive and reproduce in soils with high concentration of heavy metals, but are also able to accumulate them within their tissues. Both hyperaccumulators and halophytes face osmotic and oxidative stress and may use similar mechanisms to alleviate these stresses. One of the mechanisms employed by metal hyperaccumulators to deal with heavy metal stress is the production of compatible solutes, which serve as osmoprotectants, shielding cellular structures from damage and maintaining osmotic regulation (Lefevre et al., 2009; Schat et al., 1997; Sharma & Dietz, 2006). Halophytes also produce osmoprotectants (compatible solutes) to mitigate the effect of ion toxicity caused by salinity (Blumwald, 2000; Glenn et al., 1999; Munns & Tester, 2008). Some osmoprotectants, such as the compatible solute proline, are effective not only against salt (Flowers et al., 1977; Stewart & Lee, 1974), but can also protect plants living in soils with heavy metals like cadmium (Cd) and copper (Cu: Schat et al., 1997; Sharma & Dietz, 2006).

Because both salt and heavy metal tolerance require mechanisms to combat osmotic and oxidative stress, we might expect that species that can tolerate heavy metals might also be better at tolerating salt (Ghnaya et al., 2007; Jordan et al., 2002; Lefevre et al., 2009; Rozema & Schat, 2013). For example, some groups that contain halophytes (Table 1) are also known to contain metal hyperaccumulators [e.g., Brassicaceae (Rascio & Navari-Izzo, 2011), Asteraceae (Prasad & de Olivera Freitas, 2003)]. Future phylogenetic investigations can test whether halophytes and hyperaccumulators are found in closely related lineages, even when species that convey both types of stress resistance are discounted from the analysis.

## **Alkali Tolerance**

There are several reasons to believe that tolerance to salinity and alkalinity might be associated. First, salinity and sodicity often co-occur on the landscape (Rengasamy, 2006), so plants occupying environments that are saline and sodic need to adapt to both salinity and alkalinity (Bui, 2013). Second, these two stresses affect plants in similar ways. Both salinity and sodicity affect water use efficiency. Water uptake is hindered in sodic soils because of poor soil structure, and water loss is exacerbated because alkalinity interferes with stomatal closure through the accumulation of sodium ions (Bernstein, 1975). Also, both salinity and

alkalinity adversely affect photosynthesis (Nishiuchi et al., 2010; Yang et al., 2008). Because of these common challenges that plants face in alkaline and saline environments, similar mechanisms might be employed in dealing with both stresses (Bromham et al., 2013).

We applied a macroevolutionary comparative approach to investigate the phylogenetic association between lineages that occur in high salinity and the ones found in high alkalinity. We collated occurrence data for Australian grasses and, using geochemical modelling, we estimated the likely levels of salinity and pH that species are exposed to in their natural distributions. Using these geochemical predictions, we identified species that are likely to occur in relatively higher salinity and alkalinity. Using a taxonomic randomisation, we found that there are significantly more species than expected by chance occurring in both high predicted salinity and alkalinity (Saslis-Lagoudakis et al., submitted). Additionally, we used a complete genus-level phylogeny of grasses (Bouchenak-Khelladi et al., 2010) and plotted species occurring in high predicted salinity and alkalinity conditions on that tree. We found a non-random phylogenetic association between lineages containing the sets of taxa that occur in high predicted salinity and those predicted in high predicted alkalinity. One possible explanation of the association of species found in high predicted salinity with those from high predicted alkalinity is that some lineages have pre-existing enabling traits that makes it easier for them to adapt to either saline or alkaline environments, or both. However there are other factors, such as biogeography and the spatial overlap of salinity and alkalinity (Rengasamy, 2006) that could drive this association. Further, it is likely that the scale of geochemical predictions in that study does not reflect the variation of geochemistry in the landscape.

## **FUTURE INVESTIGATIONS OF ASSOCIATIONS BETWEEN ECOPHYSIOLOGICAL STRATEGIES**

The findings outlined in this section of the chapter suggest that salt tolerance is associated with other types of stress tolerance. However, further investigations can help us tease apart these associations. Mapping traits that potentially underlie environmental stress tolerance on phylogenies can help us explicitly test for the presence of shared mechanisms used in several abiotic tolerances (Fujita et al., 2006) within an evolutionary context. For example, investigating the evolution of a photosynthetic enzyme, a recent study showed that it was recruited in the evolution of  $C_4$  and CAM metabolism within lineages of Caryophyllales (Christin et al., 2014). Because many of the mechanisms supporting salt tolerance are involved in osmoregulation, it would be logical to assume that these mechanisms could also be involved in other types of water-use efficiency stresses, like drought and flooding (Colmer & Voisenek, 2009; Munns & Tester, 2008; Osborne & Sack, 2012; Rozema & Schat, 2013). Therefore, further studies can explore specific mechanisms involved in different water-use efficiency stresses (drought, flooding, frost, salt tolerance) in a comparative phylogenetic framework. The presence of shared mechanisms recruited in the evolution of both salt tolerance and other types of water-use efficiency stresses might partially explain the association among them.

## CONCLUSION

Salt tolerance is rare, as only 0.25% of all flowering plants are halophytes. It is a complex trait, involving several physiological, anatomical, and morphological adaptations. However, salt tolerance has been gained many times independently in several angiosperms lineages. We reveal at least 59 independent origins of salt tolerance at the family level, but the total number of origins in angiosperms is certainly much higher than this figure. Further, we find that the evolutionary dynamics of salt tolerance differ across lineages.

What could explain these different evolutionary dynamics in different lineages? Salt tolerance is associated with other ecophysiological strategies related to adaptations to harsh environments, such as C<sub>4</sub> photosynthesis, alkali, and heavy metal tolerance. One explanation for the association of salt tolerance with other ecophysiological strategies related to environmental stress is that salt tolerance builds upon a more general suite of stress-tolerance traits that provide starting points to environmental stress tolerance. Therefore, it is possible that salt tolerance is more easily acquired in lineages with particular enabling traits as starting points, and that these enabling traits also enhance the capacity of those lineages to evolve other ecophysiological strategies. However, other factors, such as biogeography, could also shape these associations. Further research is needed to explore these evolutionary patterns. Particularly the phylogenetic distribution of different anatomical and physiological traits can shed light on which traits can act as enabling traits in the evolution of salt tolerance and other types of environmental stress tolerance.

The availability of molecular phylogenies for many taxa allows us to test evolutionary hypotheses across several lineages in order to deduce general patterns of the evolution of environmental stress tolerance in angiosperms. Further, our knowledge of halophytes and plant species tolerant to other types of environmental stresses, although not complete, is richer nowadays than ever before. Additionally, methods such as geochemical modelling are being explored to test if they can predict which plants are tolerant to different types of environmental stress based on species distributions. Finally, the development of comparative phylogenetic methods in the last decade provides suitable tools to test for macroevolutionary hypotheses. All these factors make this is an exciting time to perform research in the evolution of salt tolerance, as well as other types of environmental stress.

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