

The Phylogenetic Association Between Salt Tolerance and Heavy Metal Hyperaccumulation in Angiosperms

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Abstract Salt tolerance and heavy metal hyperaccumulation are two rare plant abilities that are heavily studied for their potential to contribute to agricultural sustainability and phytoremediation in response to anthropogenic environmental change. Several observations suggest that it is worth investigating the link between the abilities to tolerate high levels of soil salinity or accumulate more of a particular heavy metal from the soil than most plants. Firstly, several angiosperm families are known to contain both salt tolerant plants (halophytes) and heavy metal hyperaccumulators. Secondly, some halophytes can also accumulate heavy metals. Thirdly, although salinity tolerance and heavy metal hyperaccumulation typically require many physiological or anatomical changes, both have apparently evolved many times in angiosperms and among closely related species. We test for a significant relationship between halophytes and hyperaccumulators in angiosperms using taxonomic and phylogenetic analyses. We test whether there are more angiosperm families with both halophytes and hyperaccumulators than expected by chance, and whether there are more species identified as both halophyte and hyperaccumulator than if the abilities were

unconnected. We also test whether halophytes and hyperaccumulators are phylogenetically clustered among species in seven angiosperm families. We find a significant association between halophytes and hyperaccumulators among angiosperm families and that there are significantly more species identified as both halophytes and hyperaccumulators than expected. Halophytes and hyperaccumulators each show low phylogenetic clustering, suggesting these abilities can vary among closely related species. In Asteraceae, Amaranthaceae, Fabaceae, and Poaceae, halophytes and hyperaccumulators are more closely related than if the two traits evolved independently.

Keywords Halophyte · Evolution · Comparative analysis · Phylogeny

Introduction

The interest in understanding the ability of some plants to tolerate harsh environments has increased due to rapid anthropogenic environmental change. A large research effort has focused on identifying plants with particular traits that can tolerate and possibly mitigate the effects of these changes (Arthur et al. 2005; Bartels and Sunkar 2005; Mahajan and Tuteja 2005; Rozema and Flowers 2008; Feuillet et al. 2008). Two common environmental changes that pose challenges for land managers in both agricultural and industrialized areas are land salinization and the contamination of soils with heavy metals. For each of these problems, a group of rare, naturally occurring plants has been identified with the potential to alleviate these problems: halophytes, salt tolerant plants, and heavy metal hyperaccumulators, plants that can extract heavy metals from the soil.

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As a consequence of common practices like land clearing and irrigation, approximately 7 % of global land surface area is salt-affected. In particular 20–50 % of irrigated agricultural land is salt-affected, which poses a significant threat to agricultural production (Munns 2005; Panta et al. 2014). Halophytes are plant species that can live in soils with salinity levels that are toxic to most plants (Glenn et al. 1999; Colmer and Flowers 2008). Halophytes are relatively rare amongst angiosperms, representing only 1–2 % of flowering plant species. They have been widely studied for their potential to contribute to the expansion and sustainability of agriculture in the wake of increasing land salinization, enabling crop production on salt-affected agricultural land as well as crop production in naturally saline areas (Glenn et al. 1999; Colmer et al. 2006; Rozema and Flowers 2008; Panta et al. 2014). Halophytes have been proposed as alternative crops for food and fodder (Weber et al. 2007; El Shaer 2010; Ventura and Sagi 2013; Ventura et al. 2015), and also for their potential ability to desalinize salt-affected soils (Ravindran et al. 2007; Rabhi et al. 2010). There has also been a large research focus on how halophytes tolerate salinity, knowledge that is being used in efforts to increase salt tolerance of established crop species (Flowers and Yeo 1995; Munns et al. 2006; Colmer et al. 2006; Rozema and Flowers 2008; Tester and Langridge 2010).

Another common consequence of anthropogenic environmental change is the contamination of soils with heavy metals (such as copper, nickel, and zinc) or metalloids (such as aluminum, arsenic, and selenium). The expansion of mining and industry has greatly increased the amount and distribution of soils contaminated with heavy metals/metalloids (Nriagu 1979), which are toxic to the vast majority of plants and pose a health risk to humans and animals. The use of some pesticides and chemical and biological fertilizers has lead to the contamination of agricultural lands with heavy metals, which can contaminate crops and fodder (Baker et al. 1994; Wuana and Okieimen 2011). Heavy metals accumulate in soils and do not dissipate over time, so it is necessary to remove or alleviate the negative effects of anthropogenic contaminants in soil and ground water.

Researchers have studied plants known as heavy metal hyperaccumulators as an alternative to chemical and physical methods of removing heavy metals from soils (Vara Prasad and de Oliveira Freitas 2003; Arthur et al. 2005). Heavy metal hyperaccumulators, referred to here as hyperaccumulators, are plant species that are able to not only tolerate but also extract large amounts of one or a few types of heavy metals from the soil into aerial tissues. Like halophytes, hyperaccumulators are rare and represent approximately 0.2 % of plant species (Baker and Brooks 1989; Rascio and Navari-Izzo 2011; Cappa and Pilon-

Smits 2014). Hyperaccumulators can take up hundreds or thousands of times greater concentrations of particular heavy metals/metalloids than most plants (Rascio and Navari-Izzo 2011), and so have been studied for their potential use in phytoremediation, using plants to clear or alleviate the effects of excess metals from contaminated soils (Arthur et al. 2005; Ali et al. 2013). Phytoremediation has been proposed as a cost-effective and environmentally low-impact alternative for removing or alleviating the effects of heavy metal contamination of soils. Many studies have focused on either the direct use of natural hyperaccumulators or on engineering novel hyperaccumulators for phytoremediation (Vara Prasad and de Oliveira Freitas 2003; Arthur et al. 2005; Manousaki and Kalogerakis 2011b). Hyperaccumulators have also been researched for use in phytomining, using plants to extract valuable metals/metalloids from contaminated and naturally-occurring metalliferous soils (Brooks et al. 1998; Anderson et al. 1999; Sheoran et al. 2009).

The large research effort focusing on hyperaccumulators and halophytes has produced experimental and observational evidence that salt tolerance and heavy metal hyperaccumulation may be physiologically and evolutionarily associated. For example, several halophytes can accumulate heavy metals, such as *Arthrocnemum macrostachyum* (Amaranthaceae) and *Tamarix smyrnensis* (Tamaricaceae) (Jordan et al. 2002; Kadukova et al. 2008; Redondo-Gómez et al. 2010; Redondo-Gómez 2013). One explanation for why some halophytes can accumulate heavy metals is that both abilities rely on similar functional mechanisms. Excess salt and heavy metals are both toxic to plants, and both salt tolerance and heavy metal hyperaccumulation are often the results of many physiological or anatomical modifications (Flowers et al. 1977; Baker and Brooks 1989). Salt and heavy metals can both induce osmotic and metabolic stresses, and halophytes and hyperaccumulators may use similar mechanisms to combat these stresses (Flowers et al. 1977; Baker and Brooks 1989; Thomas et al. 1998; Przymusinski et al. 2004). For example, one effect of toxic levels of metals and salts within plants is the increased production of reactive oxygen species (ROS; Briat and Lebrun 1999; Bose et al. 2014), which unchecked can lead to cell damage and plant death. Some halophytes and hyperaccumulators use the same mechanisms for dealing with ROS, including the production of compatible solutes, which act as osmoprotectants (Schat et al. 1997; Glenn et al. 1999; Sharma and Dietz 2006; Munns and Tester 2008; Lefèvre et al. 2009). In some cases, halophytes and hyperaccumulators produce the same osmoprotectants, like proline (Stewart and Lee 1974; Flowers et al. 1977; Schat et al. 1997; Sharma and Dietz 2006). Some halophytes and hyperaccumulators are also known to use shedding to deal with excess toxins, pushing salts and metals into leaves or other aerial tissues and then shedding them to

remove toxins (Albert 1975; Boyd 2004). Specific anatomical adaptations may also allow for some species to tolerate and remove heavy metals and salts. For example, studies have shown that specialized salt glands, which extrude excess salt out of the plant body, are also able to extrude multiple types of heavy metals/metalloids (Jordan et al. 2002; Kadukova et al. 2008; Manousaki and Kalogerakis 2011b).

In addition to the observation that some species are identified as both halophytes and hyperaccumulators, there also appears to be a broader taxonomic and evolutionary association between halophytes and hyperaccumulators among plant families. Although halophytes and hyperaccumulators are rare, they are found in a diverse range of angiosperm families. Several angiosperm families, including Asteraceae, Euphorbiaceae and Brassicaceae contain both halophyte and hyperaccumulator species (Flowers et al. 1977; Vara Prasad and de Oliveira Freitas 2003; Menzel and Lieth 2003; Rascio and Navari-Izzo 2011). One possible explanation for the co-occurrence of halophytes and hyperaccumulators in these families is that some feature of these lineages may make the evolution of salt tolerance, heavy metal hyperaccumulation, or both, more likely.

By comparing phylogenetic studies, it also appears that salt tolerance and heavy metal hyperaccumulation show some similar evolutionary patterns. Although salt tolerance and heavy metal hyperaccumulation often involve multiple physiological or anatomical mechanisms, phylogenetic and taxonomic evidence suggests that there have been many independent evolutionary origins of both salt tolerance (Flowers et al. 2010; Bennett et al. 2013; Saslis-Lagoudakis et al. 2014) and heavy metal hyperaccumulation (Cappa and Pilon-Smits 2014). Phylogenetic analyses suggest that salt tolerance has evolved many times among species within several families (Bennett et al. 2013; Moray et al. 2015). And it has also been suggested that heavy metal hyperaccumulation has evolved multiple times within some families and genera (Krämer 2010; Cecchi et al. 2010; Cappa and Pilon-Smits 2014). These observations suggest that salt tolerance and heavy metal hyperaccumulation may both evolve more often in some taxonomic groups than expected considering their rarity amongst species.

The observed association between salt tolerance and heavy metal hyperaccumulation creates an opportunity to explore whether having a particular tolerance to one environmental stress is associated with the ability to tolerate other types of stresses. One way to establish whether salt tolerance and heavy metal hyperaccumulation are associated is to use taxonomic information to find out which groups (e.g., angiosperm families) contain both halophytes and hyperaccumulators and to identify which species are identified as both a halophyte and a hyperaccumulator. But knowing whether halophytes and hyperaccumulators are

related taxonomically does not fully answer the question of whether the two abilities are closely related in an evolutionary context. Using a phylogenetic comparative approach we can test not only whether salt tolerance and heavy metal hyperaccumulation are found in the same broad groups or occur in some of the same species, but also whether halophytes and hyperaccumulators are closely related among species. For example, if halophytes and hyperaccumulators are often found in closely related lineages, this could mean that within families, some lineages are more likely to produce both types of species, and others are more likely to produce none. Understanding the evolutionary relatedness between these traits could lead to the identification of factors that support the ability to tolerate multiple harsh conditions, which could contribute to the production of novel varieties of tolerant and multi-tolerant plants for practical use (Manousaki and Kalogerakis 2011a; Hamed et al. 2013; Anjum et al. 2014; Lutts and Lefevre 2015). In this study we take an important first step towards achieving these goals by establishing whether there is a significant taxonomic association and phylogenetic relationship between halophytes and hyperaccumulators in the angiosperms.

Using lists of species identified in published sources as halophytes and hyperaccumulators, we first investigate the broader relationship between salt tolerance and heavy metal hyperaccumulation in angiosperms. We begin by asking whether there are more angiosperm families that have both halophytes and hyperaccumulators than expected. Then, using the phylogenies of seven angiosperm families, we test whether salt tolerance and heavy metal hyperaccumulation have a tendency to occur in closely related lineages by testing whether halophyte and hyperaccumulator species are more closely related than predicted by a model where each ability evolves independently. We also identify multi-tolerant species (species that are identified as both a halophyte and hyperaccumulator), and investigate whether there are more multi-tolerant species among angiosperms than expected given the rarity of both tolerances.

Methods

Taxonomic Data

We compiled lists of angiosperm species reported to be hyperaccumulators or halophytes. Both heavy metal hyperaccumulation and salt tolerance can be considered on continuous scales (for example, some species can tolerate higher concentrations of salt than others), but continuous measures of tolerance/accumulation are available for relatively few species. Since we wanted to analyze the relationship between all species known to tolerate salt or hyperaccumulate heavy metals, we had to treat each ability

as a binary character. Categorizing species as able to hyperaccumulate heavy metals or not, or as salt tolerant or not, allowed us to include a wider range of published sources, so that we could include species identified by both observational and experimental evidence. We included species identified as a halophyte or hyperaccumulator in published field studies and surveys, as well as halophytes and hyperaccumulators identified in laboratory and greenhouse experiments. We analyzed the relationship between halophytes and hyperaccumulators at the species level, so we considered a species to have the propensity to tolerate salinity or hyperaccumulate heavy metals/metalloids if one or more variety or subspecies was identified as a halophyte or a hyperaccumulator in the literature.

Heavy Metal Hyperaccumulator List

To create a list of hyperaccumulators, we searched the Web of Science (Accessed January 2012) with the term “hyperaccum*” to find published reports of angiosperm species with the ability to hyperaccumulate metals (see Supplemental Material for list of references). We included species that the authors reported as hyperaccumulators. We did not restrict our list to species able to tolerate or accumulate a specific amount of metal since this information is available for relatively few species and because measures of tolerance and accumulation can vary in different experimental conditions (Goolsby and Mason 2015). The resulting list had 593 species. We also added 54 species from a published list (Cappa and Pilon-Smits 2014). Because hyperaccumulators may be able to tolerate and take up one or a few particular heavy metals/metalloids, we recorded the elements accumulated by each species where available. However, because we treat hyperaccumulation as a binary trait, we did not take into account metal specificity in our analysis.

Halophyte List

We used a list of halophytes from Moray et al. (2015). This list included about 2600 taxa reported to grow in saline habitats (Menzel and Lieth 2003) as well as taxa from five additional published halophyte lists (Guvensen 2006; Khan and Qaiser 2006; Dagar and Gurbachan 2007; Öztürk et al. 2008; Zhao et al. 2011). The complete list contained 3468 taxa reported to be salt tolerant (including infraspecific taxa).

Association Between Halophytes and Heavy Metal Hyperaccumulators

Family-Level Taxonomic Association

In order to identify species that are reported as both hyperaccumulators and halophytes, we needed to be sure

that both lists followed a consistent taxonomy. We used the function ‘TPL’ in the R package *taxonstand* (Cayuela et al. 2012) to search for accepted names of each taxon based on The Plant List (2010) taxonomy. This search resulted in a list of 531 accepted hyperaccumulator species. After removing infraspecific epithets and comparing the list of halophytes from the literature to The Plant List (2010), we identified 2934 accepted halophyte species.

Our first aim was to investigate the observation that several angiosperm families are known to contain both halophytes and hyperaccumulators. We tested whether there were more families containing both halophytes and hyperaccumulators than expected if the two were distributed randomly with respect to each other, accounting for the total number of species in each family and the observed proportions of halophytes and hyperaccumulators among angiosperms. Using the lists of accepted hyperaccumulators and halophytes, we first identified which families had at least one hyperaccumulator and one halophyte based on The Plant List (2010) taxonomy. We included 411 angiosperm families, by checking the 413 families identified by the Linear Angiosperm Phylogeny Group III (Haston et al. 2009) against those listed on the APG III website (Stevens 2001). Two families, Aristolochiaceae and Lactoridaceae are considered one family by the APG III website and Buxaceae and Haptanthaceae are also considered synonymous (Stevens 2001). Here we considered these families as synonyms, reducing the number of angiosperm families included in this analysis from 413 to 411. We also recognized Ripogonaceae (Haston et al. 2009) as an alternative spelling of Rhipogonaceae (APG 2009). We collected an estimate of the number of species in each family, by taking the mean of the species estimates listed for each family on the APG III website (Stevens 2001). We also estimated the observed proportion of species identified as either a halophyte or hyperaccumulator among the total of 276,000 angiosperm species across all families. We compared the observed number of families with both one or more halophytes and hyperaccumulators to a Poisson binomial distribution, using the ‘ppoibin’ function in the R package *poibin* (Hong 2013), parameterized by the observed number of families identified as having at least one halophyte and one hyperaccumulator, and the probability of each angiosperm family having both a hyperaccumulator and halophyte given the observed proportions of each ability among all angiosperm species and the estimated number of species in each family.

Frequency of Multi-Tolerant Species

Next we asked whether salt tolerance and heavy metal hyperaccumulation occurred in the same species more often than expected given the rarity of both abilities. We

tested if there were more species that were included in both the lists of accepted halophyte and hyperaccumulator species than expected by chance. Using the estimates of total angiosperm species calculated in the family-level taxonomic analysis, we calculated the observed frequencies of halophytes, hyperaccumulators, multi-tolerant and non-tolerant species among angiosperm species, and the expected probabilities of each species only being a halophyte, only being a hyperaccumulator, being a multi-tolerant species, or not having either ability. We then used a X^2 test for given probabilities to ask if the observed frequency of multi-tolerant species was significantly greater than predicted by the expected probabilities.

Phylogenies

We also aimed to assess the phylogenetic relatedness between halophytes and hyperaccumulator species within families. We used a published phylogeny of over 56,000 angiosperm taxa (Smith et al. 2011) to extract species-level trees for a number of angiosperm families. In order to select informative examples for analysis, we needed to target families that had enough halophytes and hyperaccumulators to allow us to test the phylogenetic relationship between the two. We first identified family clades in the phylogeny that had six or more terminal taxa (tips) in the phylogeny matching species on the halophyte list and six or more tips matching species on the hyperaccumulator lists. We then created a family-level phylogeny for each of the seven families that met these criteria. If all tips associated with a family were monophyletic in the Smith et al. (2011) angiosperm phylogeny, we extracted all taxa associated with the family according to GenBank taxonomy. For non-monophyletic families, we only included species that fell within the main clade of the family (see Supplementary Material for list of excluded tips). In some cases, we also removed a small number of tips from the family clade that were not associated with the target family (see Supplementary Material for details). We then removed tips from the family trees that were not identified by a standardized genus-species epithet, as we could not confidently match them to the lists of halophytes and hyperaccumulators. Specifically we excluded any tips that included the taxonomic epithets “af”, “aff”, “cf”, or “sp”. We also removed any tips representing hybrid taxa, by removing tips that included one genus and two specific epithets separated by “x” or that included the word “hybrid”. We randomly resolved polytomies in the family trees using the ‘multi2di’ command in the R package *ape* (Paradis et al. 2004) since polytomies can not be analyzed using the phylogenetic metrics used in this study.

Because we analyze the relationship between halophytes and hyperaccumulators at the species level, we relabeled

the tip labels of infraspecific taxa in the family trees to the species name. Removing infraspecific epithets from tip labels sometimes resulted in multiple tips representing the same species. For each set of duplicate tips, we determined which tip had the most reliable position in the tree by choosing the tip with the most data in the published alignment that was also grouped with conspecifics and congeners. The remaining duplicates were removed from the tree. Since the Smith et al. (2011) angiosperm phylogeny does not follow The Plant List (2010) taxonomy, tips were identified as a halophyte or hyperaccumulator in the phylogenetic analyses if they matched either the accepted name identified on The Plant List (2010) or the name in the halophyte/hyperaccumulator lists, which were the names presented in the surveyed publications.

Species-Level Phylogenetic Association

Our next aim was to assess the species-level phylogenetic association between heavy metal hyperaccumulators and halophytes in different angiosperm families. The functional and taxonomic similarities between salt tolerance and heavy metal hyperaccumulation, including the observation that some species are both halophytes and hyperaccumulators, leads to the prediction that hyperaccumulator species might be quite closely related to halophytes within families. To interpret the relatedness between halophytes and hyperaccumulators, we also needed to understand the relatedness among halophytes and among hyperaccumulators. Salt tolerance has been shown to be remarkably labile in some angiosperm families, with a relatively large number of inferred independent evolutionary origins (Bennett et al. 2013; Moray et al. 2015). Several studies suggest that heavy metal hyperaccumulation has also evolved many times independently within angiosperm families (Rascio and Navari-Izzo 2011; Cappa and Pilon-Smits 2014), but the species level phylogenetic relationships have not been formally analyzed. To distinguish patterns particular to hyperaccumulators or halophytes from the relationship between the two groups, we measured phylogenetic relatedness (1) among hyperaccumulators, (2) among halophytes, and (3) between hyperaccumulator and halophyte species in a sample of angiosperm families.

Phylogenetic Relatedness Among Halophytes and Hyperaccumulators

To measure phylogenetic relatedness among halophytes and among hyperaccumulators in each angiosperm family chosen for analysis, we measured the mean nearest taxon distance (MNTD) for each group using the function ‘mntd’ in the R package *picante* (Kembel et al. 2010). MNTD (derived from nearest taxon index, NTI, Webb et al. 2002)

measures the mean phylogenetic distance between each taxon in a group to the closest relative within that group. A smaller MNTD indicates that the taxa in a group are more phylogenetically related than taxa with a larger MNTD. To assess the significance of the observed MNTD for each group (halophytes or hyperaccumulators) in each family, we compared the observed values to two null models. We first compared the observed MNTD to the MNTD values from 1000 random distributions, generated by randomly assigning tips in each family tree as either halophyte, hyperaccumulator or neither, constraining the total number of halophytes and hyperaccumulators in each randomization to the observed number in each family tree. The *p* value for each family was generated by the proportion of random comparisons with a MNTD smaller than the observed. *p* values less than or equal to 0.05 indicated that the observed MNTD was significantly smaller than 95 % of the random samples.

We then compared the observed MNTD for each group in each family to a Brownian motion (BM) model. We simulated the evolution of two independent traits, which we labeled salt tolerance and heavy metal hyperaccumulation, as continuous traits using a BM model of evolution (Felsenstein 2005; Fritz and Purvis 2010). We then converted each continuous trait to a binary one using an appropriate threshold, ensuring that the resulting number of halophyte or hyperaccumulator tips in each simulated dataset was equal to the observed numbers in each family. We repeated this process 1000 times for salt tolerance and 1000 times for heavy metal hyperaccumulation, and then measured the MNTD for each simulation. The *p* values representing phylogenetic relatedness among halophytes and among hyperaccumulators for each family was generated by the proportion of BM comparisons with a MNTD smaller than the observed. *p* values less than or equal to 0.05 indicated that the observed MNTD was significantly smaller than in 95 % of the BM simulations, suggesting that the species with that ability were more closely related on the phylogeny than expected under BM.

Phylogenetic Relatedness Between Halophytes and Hyperaccumulators

We then measured the phylogenetic distance between halophyte and hyperaccumulator species in each family phylogeny to ask whether, on average, halophytes and hyperaccumulators were more closely related to each other than expected. To do this, we used the between-community mean nearest taxon distance (BMNTD), a beta diversity metric performed using the ‘comdistnt’ function in the R package *picante* (Kembel et al. 2010). This function measures the phylogenetic distance between each taxon in one group (e.g., halophytes) and its closest relative in a second

group (e.g., hyperaccumulators), and then calculates the mean of these distances. The more closely related hyperaccumulators and halophytes are to each other, the smaller the BMNTD statistic.

Since we wanted to know about the evolutionary association between salt tolerance and heavy metal hyperaccumulation, we compared the observed BMNTD to the expected pattern under a model where salt tolerance and heavy metal hyperaccumulation evolved independently under BM. Using the simulations described above, we measured the BMNTD between one simulated halophyte distribution and one simulated hyperaccumulator distribution for each of the 1000 simulations generated for each ability. The *p* value was the proportion of simulated BM comparisons with a BMNTD smaller than the BMNTD of the observed distribution. *p* values less than or equal to 0.05 indicated that the observed BMNTD was significantly smaller than 95 % of the simulations, suggesting that halophytes and hyperaccumulators were more closely related on the phylogeny than expected if salt tolerance and heavy metal hyperaccumulation evolved independently under BM.

Results

Association Between Halophytes and Heavy Metal Hyperaccumulators

Family-Level Taxonomic Association

Of the 411 angiosperm families included in the analysis, we identified 82 families that have at least one hyperaccumulator and 149 that had at least one halophyte species (see Table S1). There were 62 families that contained both halophytes and hyperaccumulators, which is significantly more than expected by a Poisson binomial distribution parameterized by the observed proportion of halophytes and hyperaccumulators and the size of each family ($p < 0.001$). A family-level phylogenetic plot highlighting the families with halophytes and hyperaccumulators is presented in Fig. 1.

Frequency of Multi-Tolerant Species

We found that 60 species appeared on both the list of known halophyte species and the list of known hyperaccumulator species (see Table S2 for list of multi-tolerant species), representing 21 families in 15 orders (Table 1). The number of multi-tolerant species was much higher than expected based on the proportion of known halophytes and hyperaccumulators among angiosperm species (χ^2 test for given probabilities, $p < 0.001$).

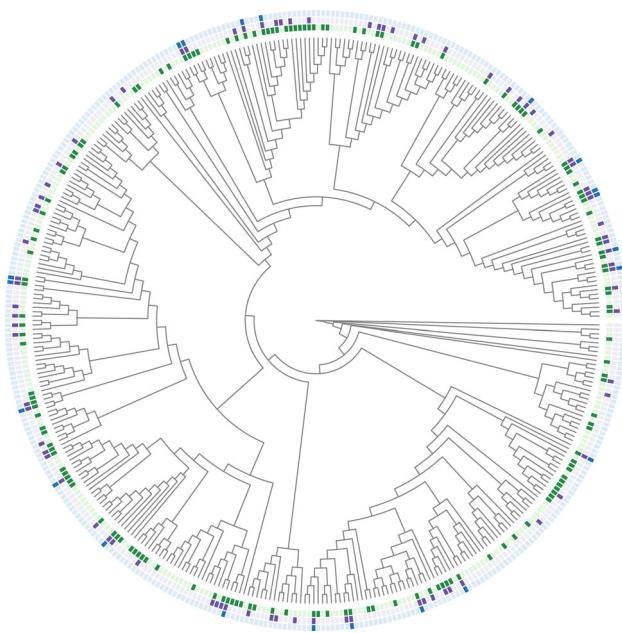


Fig. 1 Phylogeny of angiosperm families with halophytes, hyperaccumulators, and multi-tolerant species. The phylogeny contains 401 of the 411 families included in the analysis (see “Methods”) that are represented in a published phylogeny of angiosperms (Smith et al. 2011). 148 out of the 149 families with halophytes are marked in dark green, all 82 families with heavy metal hyperaccumulators are marked in dark purple, and the 21 families containing multi-tolerant species (able to tolerate salinity and hyperaccumulate heavy metals) are marked in dark blue. The family phylogeny is modified from Saslis-Lagoudakis et al. (2014). Family tip labels are presented in Figure S1. Color labels around the phylogeny were added using the ‘trait.plot’ function in the R package *diversitree* (FitzJohn 2012) (Color figure online)

Phylogenetic Relatedness Among Halophytes and Hyperaccumulators

In six of the seven families, halophytes showed low phylogenetic relatedness: halophytes were less related than expected under BM (Table 2), but more closely related than a random distribution. Similarly, heavy metal hyperaccumulators were less clustered than expected under a BM model in four of the seven families. And in another four families, hyperaccumulators were more closely related, or clustered, than expected under a random distribution. These results indicate that the phylogenetic distribution of halophytes and hyperaccumulators both have low phylogenetic relatedness in several families, but are often distinguishable from a random distribution.

Phylogenetic Relatedness Between Halophytes and Hyperaccumulators

In four of the seven families (Asteraceae, Amaranthaceae, Fabaceae, Poaceae) examined using species-level phylogenies, hyperaccumulators and halophytes were more

closely related than if the two abilities had evolved independently of each other under BM (Table 3). In the remaining three families (Brassicaceae, Euphorbiaceae, Phyllanthaceae), the phylogenetic distance between halophytes and hyperaccumulators was indistinguishable from a model where both abilities evolved independently.

Discussion

In this study, we investigated whether there is a significant taxonomic and phylogenetic relationship between the ability to tolerate soil salinity and to hyperaccumulate heavy metals from the soil. Using broad scale taxonomic approaches, we find that salt tolerance and heavy metal hyperaccumulation are significantly associated among angiosperm families, as there are more angiosperm families containing both halophytes and hyperaccumulators than expected. We also find that there are significantly more species identified as both halophytes and hyperaccumulators than expected, given the rarity of both abilities.

These findings provide evidence that there is a significant (non-random) association between salt tolerance and heavy metal hyperaccumulation in angiosperms. Furthermore, in four of the seven families that we analyzed, halophytes and hyperaccumulator species are more closely related to each other than predicted by a model of independent trait evolution, suggesting that salt tolerance and heavy metal hyperaccumulation are non-randomly distributed across lineages in these families.

The observation that more angiosperm families contain both halophytes and hyperaccumulators than expected suggests that some families are more likely to produce both halophytes and hyperaccumulators than others. By inspecting the data (Table S1), this pattern does not seem to be driven by the prevalence of multi-tolerant species that can both tolerate salinity and hyperaccumulate heavy metals. One explanation for why some families produce both types of species is that these families have underlying “enabling traits” (Edwards and Donoghue 2013) that may support the ability to tolerate excess salinity or hyperaccumulate heavy metals. For example, exposure to excess salinity and heavy metals both induce osmotic stress, so it could be that halophytes and hyperaccumulators evolve more often in families with pre-existing adaptations to other environmental stresses that induce osmotic stress such as drought or aridity. In support of this idea, there is evidence that salt tolerance evolves more often in lineages that use C₄ photosynthesis (Sage 2004; Bromham and Bennett 2014). C₄ photosynthesis is associated with increased water use efficiency in arid environments (Sage 2004), so it could be that C₄ plants can more readily tolerate osmotic stress from excess salinity (Bromham and

Table 1 Angiosperm families that include species able to tolerate salinity and hyperaccumulate heavy metals

Order	Family	Family size	Halos	Hypers	Multi
Alismatales	Araceae	4759	8	4	2
Asparagales	Iridaceae	2025	10	5	2
Asterales	Asteraceae	23,600	275	84	9
Brassicales	Brassicaceae	3710	40	92	3
Caryophyllales	Aizoaceae	2035	46	2	2
–	Amaranthaceae	2275	508	11	7
–	Plumbaginaceae	836	62	1	1
–	Polygonaceae	1110	41	7	1
Commelinales	Pontederiaceae	33	3	1	1
Fabales	Fabaceae	19,500	252	27	4
Gentianales	Apocynaceae	4555	44	2	1
Lamiales	Lamiaceae	7173	31	12	2
–	Plantaginaceae	1900	35	2	1
Malpighiales	Euphorbiaceae	5735	43	37	3
Malvales	Malvaceae	4225	56	8	2
Myrtales	Lythraceae	620	23	2	1
Poales	Cyperaceae	5430	124	8	1
–	Poaceae	11,160	345	29	14
Solanales	Convolvulaceae	1625	22	5	1
–	Solanaceae	2460	42	2	1
Zygophyllales	Zygophyllaceae	285	30	1	1

Family size is the mean of the number of estimated species from each family (Stevens 2001), halos is the number of known halophytes, hypers is the number of known heavy metal hyperaccumulators, and multi are the species that are identified to both tolerate salinity and hyperaccumulate heavy metals. A complete list of the multi-tolerant species identified is presented in Table S2

Table 2 Phylogenetic signal measured by MNTD of heavy metal hyperaccumulators and halophytes in phylogenies representing seven angiosperm families

Order	Family	Heavy metal hyperaccumulators					Halophytes				
		MNTD (Obs.)	MNTD (BM)	MNTD (BM p)	MNTD (Ran.)	MNTD (Ran. p)	MNTD (Obs.)	MNTD (BM)	MNTD (BM p)	MNTD (Ran.)	MNTD (Ran. p)
Asterales	Asteraceae	10.7	4.8	0.999	16.8	<0.001	9.5	4.1	1.000	12.2	<0.001
Brassicales	Brassicaceae	5.0	4.1	0.853	10.2	<0.001	7.6	4.9	0.953	13.4	<0.001
Caryophyllales	Amaranthaceae	14.9	6.1	0.995	14.6	0.548	3.2	2.9	1.000	3.5	<0.001
Fabales	Fabaceae	20.3	7.1	0.998	25.2	0.098	8.1	3.8	1.000	10.6	<0.001
Malpighiales	Euphorbiaceae	13.7	7.2	0.947	18.4	0.067	7.5	5.1	0.909	13.2	<0.001
–	Phyllanthaceae	4.2	5.1	0.339	10.5	<0.001	11.3	5.9	0.968	12.0	0.384
Poales	Poaceae	12.8	5.1	1.000	16.1	0.019	5.5	3.5	1.000	7.5	<0.001

MNTD was evaluated separately for hyperaccumulators and halophytes in each family. Observed MNTD is reported as well as the mean MNTD from 1000 Brownian motion (BM) and 1000 random (ran.) sets. *p* values indicate whether the observed MNTD is significantly larger (*p* > 0.95, italics) or significantly smaller (*p* < 0.05, bold) than predicted by the BM or randomized set. Bold text represents values that are significantly smaller than expected (more closely related than expected) under a particular model, and italics show that the observed value is significantly larger than expected (less closely related than expected)

Bennett 2014). Similarly, heavy metal hyperaccumulation may also be associated with drought tolerance (Proctor 1999; Hughes et al. 2001; Anacker 2014). Many hyperaccumulators are endemic to serpentine habitats, which are

often arid and experience drought conditions (Proctor 1999; Hughes et al. 2001; Anacker 2014), and experimental evidence suggests that a plant's response to drought and heavy metals are similar (de Silva et al. 2012). Some

Table 3 Results for the between-group mean nearest taxon distances (BMNTD) in phylogenetic trees representing seven angiosperm families

Order	Family	Family size	Tips in tree	Hypers in tree	Halos in tree	Multi in tree	Obs. BMNTD	BMNTD mean	BMNTD (<i>p</i>)
Asterales	Asteraceae	23,600	4361	40	100	7	16.5	35.9	0.024
Brassicales	Brassicaceae	3710	1216	45	21	2	15.2	25.6	0.085
Caryophyllales	Amaranthaceae	2275	580	8	261	6	11.2	20.0	0.001
Fabales	Fabaceae	19,500	3927	11	133	3	21.9	47.4	0.014
Malpighiales	Euphorbiaceae	5735	1030	7	18	1	17.8	27.7	0.116
–	Phyllanthaceae	1745	254	9	6	0	13.2	16.3	0.312
Poales	Poaceae	11,160	2101	24	170	12	12.2	29.8	0.001

The mean number of estimated species in each family is taken from the APG III website (Stevens 2001). The number of species in each family tree (tips in tree) is stated, along with the number of heavy metal hyperaccumulators (hypers) and halophytes (halos) in each tree, as well as the number of species that are known to be both (referred to as multi-tolerant species, multi). The observed BMNTD is listed as well as the mean BMNTD for the 1000 Brownian motion simulations of each trait (BMNTD mean). *p* values indicate whether the observed BMNTD is smaller (*p* < 0.05) than expected for a model where each trait evolves independently under BM. Bold text represents values that are significantly smaller than expected (more closely related than expected) under BM

evidence also suggests that accumulated heavy metals may even play a role in increasing drought tolerance (Bhatia et al. 2005).

By compiling and comparing lists of halophytes and hyperaccumulators, we have identified 60 species from a diverse range of angiosperm groups that are able to both tolerate salt and hyperaccumulate heavy metals. Based on the proportion of known halophytes and hyperaccumulators among angiosperms, and assuming that the two abilities are taxonomically independent, we would predict only a few angiosperm species to have both abilities. Therefore, there are many more multi-tolerant species than expected if there were no link between salt tolerance and heavy metal accumulation. The identification of significantly more multi-tolerant species than expected provides further evidence that physiological mechanisms can allow species to both tolerate salinity and hyperaccumulate heavy metals (Anjum et al. 2014). Previous work on the use of halophytes for phytoremediation of heavy metals has focused on highly salt tolerant halophytes with specialized anatomical salt glands that can also excrete heavy metals (Kadukova et al. 2008). But the 60 multi-tolerant species we identify come from a broad range of families and orders. Not all of the angiosperm orders identified are known to have species with salt glands (Flowers et al. 2010), which suggests that the ability to tolerate salt and hyperaccumulate heavy metals is not only determined by the presence of these specialized anatomical features. We hope this list of species (Table S2) will be useful in future studies into common mechanisms involved in salt tolerance and heavy metal hyperaccumulation as well as in research identifying species for phytoremediation.

We also find that in some families, halophyte and hyperaccumulator species are significantly more closely

related phylogenetically than expected if the two abilities evolved independently under BM. This pattern might indicate that in these families salt tolerance and heavy metal hyperaccumulation are more likely to evolve in the same lineages. If this is true, these families might be good targets for future studies on the evolution of multiple stress tolerance and the identification and development of halophytic-hyperaccumulator species for use in phytoremediation. However, we only find that halophytes and hyperaccumulators are significantly related in a few families, suggesting that the relationship between salt tolerance and heavy metal hyperaccumulation may not be consistent among angiosperm families. Our results may be influenced by incomplete data on halophytic and hyperaccumulating species as well as incomplete phylogenetic sampling. It is likely that more halophytes and hyperaccumulators will be identified in future, which could change our understanding of how these abilities are related. The phylogenetic tree of angiosperms used in this study (Smith et al. 2011) represents 10 % of angiosperm species, so complete sampling of angiosperm taxa would further clarify our understanding of the relationship between halophytes and hyperaccumulators.

Our results for phylogenetic relatedness among halophytes and hyperaccumulators suggest that both abilities have low phylogenetic relatedness. Inspection of the family phylogenies (Figure S2) suggests that both halophytes and hyperaccumulators are scattered across the phylogenies, rather than being clustered into a few clades containing many tolerant species, supporting previous findings that both traits may be labile amongst angiosperm species (Bert et al. 2003; Greenwood and MacFarlane 2009; Cecchi et al. 2010; Bennett et al. 2013; Cappa and Pilon-Smits 2014; Moray et al. 2015). One explanation for this pattern is that both traits can evolve over short time scales. For example,

the amount of salt that halophytes can tolerate and the amount of metal hyperaccumulators can retain can vary not only between closely related species (Bert et al. 2003; Greenwood and MacFarlane 2009; Cecchi et al. 2010; Rozema 2014), but even between populations of the same species (Antonovics et al. 1971; Wu et al. 1975; Reeves et al. 2001). Furthermore, some of the mechanisms for salt tolerance and heavy metal hyperaccumulation involve the regulation or alteration of existing functions rather than the development of novel structures like salt glands (Flowers et al. 1977; Hanikenne and Nouet 2011). If regulatory changes are more labile than anatomical features or are more likely to occur in some lineages, this could contribute to the repeated evolution of these abilities.

In this study, we have analyzed salt tolerance and the ability to hyperaccumulate heavy metals as binary characters in order to allow us to include the maximum number of species and look at broad patterns across angiosperms. If continuous measures of tolerance were available for more species, it would permit a closer examination of the links between these tolerances, and may have practical benefits. For example, identifying species that have very high salt tolerance and can also accumulate multiple types of metals may be most useful for phytoremediation of contaminated salt marshes/lakes (Redondo-Gómez et al. 2010).

Conclusions

A large research effort has focused on the use of halophytes and heavy metal hyperaccumulators for practical use. Several observations have highlighted the physiological and taxonomic association between salt tolerance and heavy metal hyperaccumulation as well as the similarities in their patterns of evolution. We confirm that there is a significant taxonomic association between salt tolerance and heavy metal hyperaccumulation in angiosperms: significantly more angiosperm families contain both halophytes and hyperaccumulators than expected and there is a significantly large number of angiosperm species that can both tolerate salinity and hyperaccumulate heavy metals. Both tolerances are scattered across the phylogenies of several families and have low phylogenetic relatedness, suggesting that salt tolerance and heavy metal hyperaccumulation may vary among closely related species. Halophytes and hyperaccumulators are significantly closely related to each other in some families, but we do not find evidence that this pattern is consistent across angiosperm families. We hope that the identification of families with a significant association between salt tolerance and heavy metal hyperaccumulation and the identification of a large and diverse set of multi-tolerant species will contribute to

future advances in phytoremediation and agricultural sustainability.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest associated with this research.

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