Salt tolerance evolves more frequently in C₄ grass lineages

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Abstract
Salt tolerance has evolved many times in the grass family, and yet few cereal crops are salt tolerant. Why has it been so difficult to develop crops tolerant of saline soils when salt tolerance has evolved so frequently in nature? One possible explanation is that some grass lineages have traits that predispose them to developing salt tolerance and that without these background traits, salt tolerance is harder to achieve. One candidate background trait is photosynthetic pathway, which has also been remarkably labile in grasses. At least 22 independent origins of the C₄ photosynthetic pathway have been suggested to occur within the grass family. It is possible that the evolution of C₄ photosynthesis aids exploitation of saline environments, because it reduces transpiration, increases water-use efficiency and limits the uptake of toxic ions. But the observed link between the evolution of C₄ photosynthesis and salt tolerance could simply be due to biases in phylogenetic distribution of halophytes or C₄ species. Here, we use a phylogenetic analysis to investigate the association between photosynthetic pathway and salt tolerance in the grass family Poaceae. We find that salt tolerance is significantly more likely to occur in lineages with C₄ photosynthesis than in C₃ lineages. We discuss the possible links between C₄ photosynthesis and salt tolerance and consider the limitations of inferring the direction of causality of this relationship.

Introduction
The amount of salt-affected land, currently over 6% of the land surface area, is increasing through agricultural practices and land clearance. Irrigated land, which produces a third of the world’s food, is particularly prone to salinization: between 20 and 50% of the world’s irrigation schemes are salt-affected (Flowers, 2004; Flowers et al., 2010; Munns, 2011). Understanding the evolution and maintenance of salt tolerance in plants may help to develop strategies for utilizing and managing salt-affected land.

There are over 350 naturally halophytic (salt tolerant) grass species and subspecies. These halophytic grasses are not clustered in clades of related species, all descended from a few independent origins of salt tolerance. Instead, salt tolerance has evolved frequently in a large number of different lineages. A recent study estimated that there have been over 70 independent origins of salt tolerance in the grass family (Bennett et al., 2013). Yet, although there is considerable benefit to producing crop plants that can grow on salt-affected land (Glenn et al., 1999; Rozema & Flowers, 2008), there have been few commercially viable salt-tolerant cereal crops produced (Flowers & Yeo, 1995; Flowers & Flowers, 2005).

Why has it been so difficult to breed salt tolerance into cereal crops when it has evolved so many times within the grass family? There are several possible explanations (which are not mutually exclusive). Firstly, it may be that salt tolerance is a physiologically costly trait so that it is difficult to develop a productive crop plant that can produce commercially viable yields while dealing with environmental salt. Secondly, salt tolerance is a genetically complex trait, which may not present an easy target for breeding programs or genetic engineering.
manipulation (Roy et al., 2011). Thirdly, salt tolerance may be more easily acquired with particular back-
grounds as starting points, and thus will evolve more
easily in certain lineages that already have these traits.
It is this third possibility that we wish to examine in
this study.

One possible background trait that may enhance
the capacity to evolve salt tolerance is photosynthetic path-
way. The C₄ mechanism of carbon fixation is a modi-
fied version of the ancestral (C₃) photosynthetic pathway,
and it has evolved independently over 60 times in angiosperms (Sage et al., 2012), including an
estimated 22–24 gains within the grass family (Edwards & Smith, 2010; Grass Phylogeny Working Group II,
2012). By increasing the efficiency of carbon fixation,
C₄ plants can reduce photorespiration and thus allow
higher water-use efficiency and productivity. Therefore,
C₄ photosynthesis has been assumed to have advanta-
ges under conditions that promote photorespiration,
such as heat, drought, salinity and low atmospheric
CO₂ (Sage & Monson, 1999; Sage, 2004; Sage et al.,
2012; Christin et al., 2013).

Plants with C₄ photosynthesis are often found in salt-
affected areas, and taxa with C₄ carbon fixation appear
to be overrepresented among halophytes (Aronson,
1989; Sage & Monson, 1999; Dajic, 2006; Eallonardo et al., 2013). However, the association between photo-
synthetic pathway and salt tolerance needs to be for-
mally tested within a phylogenetic framework in order
to account for confounding factors (Christin et al.,
2009; Osborne & Freckleton, 2009; Taylor et al., 2010).
C₄ species are nonrandomly distributed in the grass
phylogeny (Table 1), with all known C₄ species occur-
ing in the large ‘PACMAD’ clade, which contains the
subfamilies Panicoideae, Arundinoideae, Chloridoideae,
Micrairoideae, Aristidoideae and Danthonioideae (Christ-
in et al., 2009, 2013). So even if a disproportionate
number of halophytic grasses use C₄ photosynthesis,
it is unclear whether this is due to a specific association
between the two traits, or because there is some other
feature of the PACMAD clade that increases the likeli-
hood of evolving salt tolerance (Edwards et al., 2007).

Here, we ask whether C₄ photosynthesis is specifically
associated with the evolution of salt tolerance, in order
to shed light on some of the factors that have allowed
some grass lineages to adapt to saline environments.

Materials and methods
A list of halophytic grasses was taken from Bennett et al. (2013). Most studies use a standard definition of a
halophyte as any species that can successfully complete
its life cycle in saline conditions similar to those
encountered in the natural environment, where saline
conditions are defined as those where the soil solution
has an electrical conductivity equivalent to ~80 mM
NaCl at saturation, following Aronson (1989). However,
this precise definition can rarely be applied in practice
as the exact level of tolerance is typically known only
for species that have been closely studied in the labora-
tory (Flowers, 2004). In most cases, it is necessary to
rely on reports of populations growing in saline condi-
tions in the field (see Bennett et al., 2013). For exam-
ple, the eHALOPH database (http://www.sussex.ac.uk/
affiliates/halophytes) lists some species with specific
ranges of soil electrical conductivity, but other species
according to discrete categories such as xerohalophyte
(e.g. inland salt desert species) or hydrohalophyte (e.g.
tidal swamp or salt marsh species).

We used the molecular phylogeny published by
Edwards and Smith (2010), which includes 2684 taxa
(approximately 20% of all grass species). Two hundred
of these taxa were identified as halophytes, following
Bennett et al. (2013). To test the generality of patterns,
all analyses were run both on the phylogeny of all Poa-
ceae and also on a subtree containing the PACMAD
clade only. Information on the photosynthetic path-
ways of all grasses in the phylogeny was also taken
from Edwards and Smith (2010). To test that our
results are not the result of sampling bias in Edwards
and Smith data set, we also combined a list of all grass
genera containing halophytes (see Bennett et al., 2013;
Table S2) with the complete genus-level phylogeny
and photosynthetic pathway data set of Bouchenak-Khelladi
et al. (2010).

A randomization test was conducted to test whether
more halophytes occur in C₄ clades than expected by
chance. A null distribution of the expected number of
halophytes occurring in C₄ lineages was generated by
randomly reassigning character states (200 halophytic/
2484 nonhalophytic) across the tips of the phylogeny,
then counting the number of these that fell on C₄ taxa.
The randomization was repeated 10 000 times. We
then compared the observed number of halophytes in
C₃ and C₄ clades to this null distribution. The associa-
tion between photosynthetic pathway and salt toler-
ance was deemed to be significantly different from
chance when the observed number of halophytes was
greater or less than in 95% of randomizations.

Table 1 The halophytes include in this study, as a proportion
of the number of species represented in the phylogeny of Edwards
and Smith (2010). There are proportionally more halophytes in
the PACMAD clade (Panicoideae, Arundinoideae, Chloridoideae,
Micrairoideae, Aristidoideae and Danthonioideae), which contains
both C₃ and C₄ taxa, than there are in the BEP clade
(Bambusoideae, Ehrhartioideae, Pooidae), which contains only
C₃ lineages.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Species</th>
<th>Halophytes</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEP</td>
<td>C₃ only</td>
<td>1526</td>
<td>87</td>
</tr>
<tr>
<td>PACMAD</td>
<td>C₃ and C₄</td>
<td>1143</td>
<td>118</td>
</tr>
</tbody>
</table>
The correlation between salt tolerance and photosynthetic pathway was tested using Pagel's (1994) correlation analysis for discrete characters, as modified by Maddison and Maddison (2006). We analysed the transition rate between two states for the two characters that is between salt tolerant and salt sensitive, and between C3 and C4. This method estimates the fit of a Markov model where the rate of change in each character is independent of the state of the other and compares it to the fit of a correlated, state-dependent model, where the rate of transition from salt sensitive to salt tolerant is dependent on the type of photosynthetic pathway. If the state-dependant model fits significantly better, then this suggests that the evolution of the two traits is correlated. The significance of the likelihood difference between the models is estimated by comparison with simulated data. We used the 'Pagel94 correlation analysis' function of mesquite, optimizing likelihoods with ten iterations and estimating significance from 1000 simulations (Maddison & Maddison, 2006) using a maximum likelihood omnibus test as described by Pagel (1994). The optimal scaling of the kappa parameter was selected with the best fit to equal branch lengths (see Bennett et al., 2013). The rate of evolution of salt tolerance in C3 and C4 clades was compared using the estimated rates of these transitions.

**Results**

The phylogenetic distribution of salt tolerance and C4 photosynthesis is shown in Fig. 1. Significantly more halophytes occur in C4 lineages than if salt tolerance was random with respect to photosynthetic pathway, both across the whole Poaceae, and within the PACMAD clade (Table 2). For example, there are only three identified C3 halophytes in the PACMAD clade of the species-level phylogeny, where we would expect 20–60 halophytes to occur in the C3 lineages of the PACMAD if salt tolerance was randomly distributed on the phylogeny (Fig. 2).

Because not all grass species are included in our analysis, we checked that our results were not an artefact of undersampling halophytes in C3 clades. Using the list of all grass genera containing halophytes

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**Fig. 1** Distribution of halophytes (salt-tolerant species) on the grass family, mapped onto the evolutionary pattern of C4 photosynthesis estimated by Edwards and Smith (2010).

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(Bennett et al., 2013) and the complete genus-level phylogeny and photosynthetic pathway data set of Bouchenak-Khelladi et al. (2010), we found only one C₃ halophytic species from the PACMAD that was not included in our species-level analysis (Rytidosperma rufum), but many more C₄ halophytes not in the species-level phylogeny. This suggests that undersampling of C₃ halophytes has not biased this analysis.

If the overrepresentation of halophytes in the PACMAD clade was due to some feature of PACMAD other than photosynthetic pathway, then we would expect to observe many salt-tolerant C₃ species in the PACMAD. However, there are significantly fewer C₃ halophytes in the PACMAD (observed = 3) than expected on the basis of chance (expected > 20; Fig. 2). Instead, almost all C₃ halophytes on the phylogeny are found in the BEP clade (Bambusoidae, Ehrhartioideae, Pooidae), particularly in the core Pooidae. Therefore, we can conclude that PACMAD lineages with C₄ photosynthesis are more likely to contain salt-tolerant species than C₃ lineages in the PACMAD, a pattern confirmed by the correlation analyses.

The correlation analyses indicated a significant association between salt tolerance and C₄ photosynthesis on the species-level phylogeny of grasses (Table 2). The correlated model, which allows the rate of transition from salt sensitive to salt tolerant to vary according to the state of the photosynthetic pathway, fits the data significantly better than the uncorrelated model, for both the whole Poaceae family (ΔlnL = 23.7, $P < 0.001$) and for the PACMAD clade (ΔlnL = 28.0, $P < 0.001$). For the phylogeny of all Poaceae, the best-fitting model gave an estimate of the rate of gain of salt tolerance in C₄ clades that was approximately three times higher than the estimated rate of gain of salt tolerance in C₃ clades. The analysis on only the PACMAD clade gave even more pronounced results, with an estimated rate of gain of salt tolerance in C₄ clades approximately seven times higher than the estimated rate of gain of salt tolerance in C₃ clades (Table 2).

**Discussion**

Although it has often been proposed that plants with C₄ photosynthesis are more likely to be able to adapt to live in saline habitats, this hypothesis has not been robustly tested before. Using a broad-scale comparative approach, we show that halophytic grasses are significantly more likely to occur in lineages with C₄ photosynthesis than expected if salt tolerance was random with respect to photosynthetic pathway. Salt tolerance appears to have evolved repeatedly within many C₄ grass clades, with salt tolerance arising at a more frequently in C₄ lineages than in C₃ groups. In fact, salt tolerance has evolved relatively rarely in C₃ lineages outside of the ‘core’ Pooidae (Edwards & Smith, 2010). But the observation of this significant correlation does not, by itself, establish a direct causal connection between the two traits, nor the direction of causality. Does salt tolerance favour the evolution of C₄ photosynthesis, does C₄ promote the evolution of salt tolerance, or are both traits linked indirectly, for example, by tending to co-occur in taxa adapted to open and arid habitats?

Taken at face value, our results could be interpreted as evidence that, within the grass family, C₃ lineages have been more likely to develop salt tolerance than C₄ lineages. There are far more origins of salt tolerance within the grasses (around 70) than origins of C₄ (around 20), and the gains of salt tolerance tend to be distributed near the tips of the phylogeny and are therefore relatively recent (Bennett et al., 2013). This pattern is compatible with the hypothesis that the adoption of C₄ photosynthesis allowed expansion into arid and saline habitats (Stromberg, 2011); therefore, C₄ lineages were more likely to produce halophytic species (Osborne & Freckleton, 2009). C₄ lineages may be an advantageous starting point for the evolution of salt tolerance, given that the greater water-use efficiency of C₄ photosynthesis lowers the flux of water and salts through the plant per growth unit, which can reduce the amount of salt that a plant has to exclude, compartmentalize, or secrete for a given amount of carbon fixation (Sage, 2001).

An alternative explanation of this link between photosynthetic pathway and salt tolerance is that lineages adapted to saline environments may be more likely to evolve C₄ photosynthesis. It has been argued that the adaptation to harsh environments, such as arid or saline habitats, has promoted selection for C₄ photosynthesis, by favouring traits that reduce ionic stress through decreasing transpiration rates. For example, Kadereit et al. (2012) estimated that there have been 10 origins of C₄ photosynthesis in the Chenopodiaceae,
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a clade of the Amaranthaceae containing many halophytes, but probably only one ancestral origin of salt tolerance. However, as with this study, inference of direction of causality may be conflated with differences in lability between traits.

Although we can be confident of a significant evolutionary link between salt tolerance and C₄ photosynthesis, the direction of the relationship cannot be easily inferred from the phylogenetic pattern alone. This is because different patterns of trait lability could create the false impression of a directional causal relationship. If salt tolerance is relatively labile in grasses (see Bennett et al., 2013), then although most extant halophytic lineages have relatively recent origins, we can expect that salt tolerance has been gained and lost throughout the history of the grasses. Because we cannot directly reconstruct past evolution and loss of salt tolerance, we cannot rule out that C₄ photosynthesis has typically arisen in lineages growing under saline conditions, then some of those C₄ lineages lose salt tolerance or move to different habitats. So the order of acquisition may be an artefact of trait lability: C₄ photosynthesis may appear deeper in the tree, and therefore to have been gained first, because it evolves less often and is lost less often than salt tolerance.

Regardless of the direction of causality of the link between photosynthetic pathway and salt tolerance, these two hypotheses – C₄ promotes evolution of salt tolerance vs salt tolerance promoting evolution of C₄ – are not mutually exclusive. Ongoing adaptation to allow exploitation of open, arid and saline habitats may have resulted in the promotion of both salt tolerance and C₄ photosynthesis. Although responses to salinity are distinct from responses to aridity, mechanisms of tolerance to these stresses have much in common (Munns, 2002; Des Marais & Juenger, 2010), so it is possible that adaptation to aridity provides enabling conditions that promote salinity tolerance (or vice versa).

It is also possible that an indirect link between C₄ and salt tolerance could arise through ecological preference or biogeographic patterns. C₄ grasses in the PACMAD are more frequently found in open and arid habitats than C₃ PACMAD species (Osborne & Freckleton, 2009; Pau et al., 2012). As highly saline soils do not generally support closed-canopy vegetation (mangrove forests being a notable exception), halophytes will also tend to occur in open habitats. Saline soils are also particularly prevalent in arid and semi-arid regions, so the large number of halophytes in C₄ clades may be explained by their inhabiting the general areas where salinity is more prevalent. Edwards and Donoghue (2013) point out that although the biased frequencies of transitions to C₄ across the grasses may be due to anatomical enablers, because the large bundle sheath cells of PACMAD grasses could give them a natural advantage over in evolving C₄ photosynthesis, it may

Fig. 2  Distribution of expected numbers of halophytes in each clade if salt tolerance occurred independently of photosynthetic pathway. In each case, the observed value is not contained in the distribution of expected values, so the null model of chance association between salt tolerance and photosynthetic pathway can be rejected. The test was repeated on both the whole grass phylogeny and on the subclade containing the PACMAD families (see Materials and methods for details). Because all C₄ halophytes occur within the PACMAD clades, the observed number of C₄ halophytes is the same for both the whole family and the PACMAD.


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be also the case that Pooid grasses tend to be distributed in cooler climates, and there is less advantage to evolving C₄ photosynthesis than there is for the PACMAD lineages (Edwards & Still, 2008; Edwards & Donoghue, 2013).

However, adaptation to open, arid conditions does not itself appear to be sufficient for a group to develop salt tolerance. For example, the subfamily Danthonioideae contains nearly 300 species of tussock and pampas grasses, found mainly in the Southern Hemisphere (Linder et al., 2010). Many species in this subfamily are found in open and relatively arid habitats (Bouchenak-Khelladi et al., 2010; Edwards & Smith, 2010), but the subfamily shows a paucity of halophytes. Across the angiosperms, there are other examples of arid-adapted groups that have rarely evolved salt tolerance, such as the Proteaceae and Cactaceae (Flowers et al., 2010). Conversely, not all halophytes occur in open, arid environments, for example plants adapted to coastal salt marshes and mangrove forests. However, the potential for reduced transpiration rates in C₄ plants may be an advantage in salt-affected habitats even where water is not limited, as it may limit the physiological stress of osmotic adjustment.

It is interesting to note that some C₄ plants require small amounts of Na⁺ for growth (Brownell & Crossland, 1972), and so do not thrive in the absence of Na⁺ (Subbarao et al., 2003). Some C₄ plants can use sodium ions as osmoticum to allow rapid grown under saline conditions (Kronzucker et al., 2013). Sodium ions can also play a role in the concentration of CO₂ in C₄ physiology through Na⁺-coupled pyruvate transport in chloroplasts (Furumoto et al., 2011). However, the requirement for Na⁺ for growth is not universal in C₄ plants, and some C₄ grasses, including maize and sugarcane, show no growth benefits from presence of sodium (Subbarao et al., 2003).

Further studies are required to tease apart the inter-correlation of aridity, salinity, C₄ photosynthesis and salt tolerance. It would also be interesting to see whether C₄ photosynthesis is more commonly associated with the evolution of particular strategies of salt tolerance. For example, salt-tolerant grasses may rely more heavily on salt exclusion than dicotyledonous halophytes (Glenn et al., 1999), and it could be that C₄ photosynthesis is particularly beneficial to this strategy of tolerance and less beneficial to other strategies such as salt accumulation. This could be tested by finding the correlation between specific salt tolerance traits and the C₄ pathway, both within the grass family, as well as in other families.

The significant correlation between C₄ and salt tolerance in naturally occurring grass species suggests that C₄ photosynthesis may provide advantages to the development of plant varieties that can grow in salt-affected areas. It is interesting to contrast the evolutionary and agricultural development of salt tolerance and C₄ photosynthesis. There is a growing effort to engineer the C₄ pathway into C₃ crop species to increase their yields (Sage & Zhu, 2011; von Caemmerer et al., 2012). Hibberd et al. (2008) stated that ‘although generating C₄ rice is extremely ambitious, the polyphyletic evolution of C₄ photosynthesis provides cause for optimism’. The evolutionary lability of both C₄ photosynthesis and salt tolerance in grasses, and the apparent link between the two, might be considered encouraging, even if engineering C₄ photosynthesis or salt tolerance in commercially viable crops may be more difficult than some have hoped (e.g. Flowers, 2004; Flowers & Flowers, 2005; Zhu et al., 2010).

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