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Sodium hyperaccumulators in the Caryophyllales are characterized by both abnormally large shoot sodium concentrations and $[Na]_{shoot}/[Na]_{root}$ quotients greater than unity

Konrad Neugebauer[1,2,*], Martin R. Broadley[2], Hamed A. El-Serehy[3], Timothy S. George[1], Neil S. Graham[2], Jacqueline A. Thompson[1], Gladys Wright[1] and Philip J. White[1,4,*]

[1]Ecological Sciences Department, The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK, [2]Plant and Crop Sciences Division, University of Nottingham, Sutton Bonington, Loughborough LE12 5RD, UK, [3]Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia and [4]King Saud University, Riyadh 11451, Saudi Arabia
**For correspondence. E-mail konrad.neugebauer@hutton.ac.uk, philip.white@hutton.ac.uk*

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• **Background and Aims** Some Caryophyllales species accumulate abnormally large shoot sodium (Na) concentrations in non-saline environments. It is not known whether this is a consequence of altered Na partitioning between roots and shoots. This paper tests the hypotheses (1) that Na concentrations in shoots ($[Na]_{shoot}$) and in roots ($[Na]_{root}$) are positively correlated among Caryophyllales, and (2) that shoot Na hyperaccumulation is correlated with $[Na]_{shoot}/[Na]_{root}$ quotients.
• **Methods** Fifty two genotypes, representing 45 Caryophyllales species and 4 species from other angiosperm orders, were grown hydroponically in a non-saline, complete nutrient solution. Concentrations of Na in shoots and in roots were determined using inductively coupled plasma mass spectrometry (ICP-MS).
• **Key Results** Sodium concentrations in shoots and roots were not correlated among Caryophyllales species with normal $[Na]_{shoot}$, but were positively correlated among Caryophyllales species with abnormally large $[Na]_{shoot}$. In addition, Caryophyllales species with abnormally large $[Na]_{shoot}$ had greater $[Na]_{shoot}/[Na]_{root}$ than Caryophyllales species with normal $[Na]_{shoot}$.
• **Conclusions** Sodium hyperaccumulators in the Caryophyllales are characterized by abnormally large $[Na]_{shoot}$, a positive correlation between $[Na]_{shoot}$ and $[Na]_{root}$, and $[Na]_{shoot}/[Na]_{root}$ quotients greater than unity.

Key words: Angiosperm, Caryophyllales, evolution, ionome, matK phylogeny, mineral composition, shoot and root partitioning, sodium (Na) hyperaccumulation.

INTRODUCTION

Saline soils are defined as soils with an electrical conductivity (ECe) of at least 2 dS m^{-1} (White *et al.*, 2017) to 4 dS m^{-1} (Munns and Tester, 2008; Munns *et al.*, 2020), which is equivalent to 20 mM sodium chloride (NaCl) to 40 mM NaCl, respectively. Although saline soils are generally dominated by large concentrations of NaCl, they can have large concentrations of other mineral elements, including calcium (Ca), magnesium (Mg) and sulphur (S) (Boon and MacIntyre, 1968; del Carmen Martínez-Ballesta *et al.*, 2008; White *et al.*, 2017). Saline soils are widespread in dry environments, and NaCl in the rhizosphere impairs the uptake of water by a plant by lowering the osmotic potential of the soil solution (Arora and Dagar, 2019). Once taken up by a plant via its roots, Na can inhibit the activity of enzymes in the cytoplasm and become toxic (Flowers *et al.*, 2015).

Halophytes are defined as plants that can complete their life cycle in the presence of large concentrations of NaCl in the rhizosphere, for example 80 mM NaCl (Santos *et al.*, 2016) or 200 mM NaCl (Flowers *et al.*, 2015), or as plants that tolerate large concentrations of Na and Cl in their shoots (Flowers *et al.*, 2015). The growth of most crop species is inhibited when grown on soils with an ECe of 4 dS m^{-1} or even less (Munns, 2005; Arora and Dagar, 2019), and less than ~0.25 % of all angiosperm species can complete their life cycle when exposed to 200 mM NaCl (Flowers *et al.*, 2010).

Halophytism has evolved repeatedly in angiosperms (Flowers *et al.*, 2010; Bromham, 2015) and angiosperm species have developed various mechanisms for tolerating Na in the rhizosphere (Subbarao *et al.*, 2003; Munns, 2005). Species that take up Na readily and accumulate large concentrations of Na safely in their shoots ($[Na]_{shoot}$), for example *Beta vulgaris* L. (Caryophyllales), have been termed 'accumulators' (White *et al.*, 2017). Other species, for example *Triticum aestivum* L. (Poales), limit the uptake of Na from the rhizosphere into roots and the subsequent transport of Na from roots to shoots. These species have been termed 'excluders' (White *et al.*, 2017).

Although some species exhibiting C_4 photosynthesis require Na, it is not considered an essential mineral nutrient for plants in general (Broadley *et al.*, 2012), and Na deficiency does not occur in natural environments (Subbarao *et al.*, 2003; Pilon-Smits *et al.*, 2009). The compartmentalization of Na

and Cl into vacuoles enables plants to avoid toxic effects of large Na^+ and Cl^- concentrations in plant tissues and lowers the osmotic potential of vacuoles. The lowered osmotic potential of vacuoles can be used for osmotic regulation by plants and can thus be beneficial to plants growing in saline or dry environments (Glenn and O'Leary, 1984; Flowers *et al.*, 2015; Munns *et al.*, 2020).

The Caryophyllales order contains the largest number of halophytic species (n = 74 species) of all angiosperm orders, totalling >21 % of all known halophytes (Flowers *et al.*, 2010). Many Caryophyllales, for example cacti, are adapted to saline or dry environments, and ancestors of extant Caryophyllales are thought to have evolved in dry mineral-rich environments (Cuénoud *et al.*, 2002). Some Caryophyllales species have abnormally large $[Na]_{shoot}$ (>4 mg g^{-1} dry weight [DW]) when grown in non-saline conditions (Broadley *et al.*, 2004; White *et al.*, 2017).

The trait of abnormally large $[Na]_{shoot}$ among 61 Caryophyllales species from ten families grown in the same non-saline environment was defined by White *et al.* (2017) and the evolution of abnormally large $[Na]_{shoot}$ among these families has been explored previously. Recently, Ievinsh *et al.* (2021) defined Na hyperaccumulation for plant species growing in saline coastal habitats along the Baltic Sea. Although previously identified Caryophyllales species with abnormally large $[Na]_{shoot}$ (White *et al.*, 2017) also had the largest $[Na]_{shoot}$ in the study of Ievinsh *et al.* (2021), it appeared that the threshold for Na hyperaccumulation in shoots might differ between non-saline and saline environments. The association between abnormally large $[Na]_{shoot}$ and the partitioning of Na between shoots and roots has not been examined. The following four hypotheses were tested in this study:

Hypothesis 1: Caryophyllales species grown hydroponically in non-saline solution can be attributed a 'normal' or 'abnormally large' $[Na]_{shoot}$ phenotype as suggested by White *et al.* (2017).

Hypothesis 2: $[Na]_{shoot}$ is positively correlated with $[Na]_{root}$ among Caryophyllales grown in the same environment.

Hypothesis 3: The $[Na]_{shoot}/[Na]_{root}$ quotient is correlated with shoot Na hyperaccumulation among Caryophyllales species grown hydroponically in non-saline conditions.

Hypothesis 4: Observations made for Caryophyllales grown hydroponically in non-saline conditions can be generalized for Caryophyllales growing in other environments.

MATERIALS AND METHODS

Experimental conditions

A glasshouse experiment was conducted between September 2016 and January 2017 at The James Hutton Institute (UK; 56°27′24.6″N, 3°04′09.7″W). The experiment was performed on 52 angiosperm genotypes (Table 1) representing 45 Caryophyllales species and species representing four other angiosperm orders: *Brassica oleracea* L. (Brassicaceae; Brassicales), *Helianthus annuus* L. (Asteraceae; Asterales), *Hordeum vulgare* L. (Poaceae; Poales) and *Phlomis lychnitis* L. (Lamiaceae; Lamiales). The 45 Caryophyllales species represented 42 genera and 13 families, and included four genotypes of *Beta vulgaris* L. (beetroot, chard, sea beet, sugar beet).

TABLE 1. *Species grown hydroponically and their taxonomic affiliations. Species marked with an asterisk were grown hydroponically in both this study and in the experiments reported by White et al. (2017)*

Order	Family	Number of genera	Number of species	Species
Asterales	Asteraceae	1	1	*Helianthus annuus**
Brassicales	Brassicaceae	1	1	*Brassica oleracea**
Poales	Poaceae	1	1	*Hordeum vulgare**
Lamiales	Lamiaceae	1	1	*Phlomis lychnitis*
Caryophyllales	Aizoaceae	7	7	*Bergeranthus vespertinus*, *Carpobrotus edulis**, *Delosperma cooperi**, *Dorotheanthus bellidiformis**, *Lampranthus* spp., *Pleiospilos nelii*, *Tetragonia tetragonioides*
Caryophyllales	Amaranthaceae	4	7	*Amaranthus caudatus**, *Amaranthus cruentus**, *Amaranthus tricolor*, *Atriplex halimus*, *Atriplex hortensis**, *Beta vulgaris**, *Salicornia europaea*
Caryophyllales	Basellaceae	1	1	*Basella alba*
Caryophyllales	Caryophyllaceae	10	10	*Agrostemma githago**, *Cerastium tomentosum**, *Dianthus glacialis*, *Gypsophila pacifica*, *Herniaria glabra*, *Melandrium keiskei*, *Petrorhagia prolifera*, *Sagina subulata**, *Silene armeria**, *Stellaria media*
Caryophyllales	Montiaceae	3	3	*Claytonia perfoliata*, *Montiopsis umbellata*, *Phemeranthus teretifolius*
Caryophyllales	Nyctaginaceae	1	1	*Mirabilis longiflora*
Caryophyllales	Petiveriaceae	1	1	*Petiveria alliacea*
Caryophyllales	Phytolaccaceae	1	1	*Phytolacca americana**
Caryophyllales	Plumbaginaceae	4	4	*Armeria maritima**, *Limonium sinuatum**, *Plumbago auriculata**, *Psylliostachys suworowi**
Caryophyllales	Polygonaceae	7	7	*Antigonon leptopus*, *Emex australis*, *Eriogonum arborescens*, *Fagopyrum esculentum*, *Persicaria capitata**, *Rheum palmatum**, *Rumex sanguineus*
Caryophyllales	Portulacaceae	1	1	*Cistanthe grandiflora*
Caryophyllales	Simmondsiaceae	1	1	*Simmondsia chinensis*
Caryophyllales	Talinaceae	1	1	*Talinum paniculatum*

Seeds were sourced from commercial suppliers (Supplementary Data Table S1) and germinated on germination paper (Whatman, Little Chalfont, UK) soaked with deionized water in Petri dishes. The germination conditions (exposure to light, temperature) were chosen according to species requirements. Seedlings were transplanted to rockwool plugs (2.5 × 2.5 × 4 cm; Grodan, Hedehusene, Denmark) as soon as radicles were observed. Rockwool plugs were placed in plastic trays in the glasshouse in which the experiment was conducted and irrigated with tap water containing 0.14 mM Na. Rockwool plugs with established seedlings were transferred into a nutrient film technique (NFT) hydroponic system, similar to the one described by Broadley *et al.* (2003), 3–5 d after the germination of seeds. The recirculating nutrient solution contained 2 mM $Ca(NO_3)_2$, 2 mM NH_4NO_3, 0.75 mM $MgSO_4$, 0.5 mM KOH, 0.25 mM KH_2PO_4, 0.1 mM FeNaEDTA, 30 μM H_3BO_3, 25 μM $CaCl_2$, 10 μM $MnSO_4$, 3 μM $CuSO_4$, 1 μM $ZnSO_4$ and 0.5 μM Na_2MoO_4 and was replaced regularly according to plant growth rates. The pH of the nutrient solution was adjusted daily to pH 6–7 using 0.5 M KOH or 0.5 M H_2SO_4.

The hydroponic system comprised two groups of four flat-bottomed gullies (10 cm width, 4.5 cm height, 6 m length, angle ~1°) made of polyvinyl chloride (PVC). Gullies of the same group were spaced 8.5 cm apart, with 40 cm space between the two groups of gullies. A fine fleece mesh was placed at the bottom of each gully to create an even nutrient film. Flat PVC strips were mounted on top of each gully. For each gully, 90 circular holes of 3.5 cm diameter were cut into the PVC strips, 3 cm apart, to hold the rockwool plugs. The holes were covered with small PVC strips when not occupied by a rockwool plug. The recirculating nutrient solution was held in two 200-L tanks, one for each group of four gullies, and pumped evenly into the gullies. The glasshouse was set to maintain 22 °C day and 18 °C night temperatures with a day length of 16 h using automatic venting, supplementary heating and additional lighting as described by White *et al.* (2017).

The experimental design was a randomized block design with each gully representing two blocks. Up to eight individual plants per genotype were grown in each set of four gullies. One replicate per gully × block combination was achieved. More replicates of genotypes that grew slowly, and for which additional seedlings were available, were grown in the hydroponic system. All plants were harvested during their vegetative growth phase. Plants were harvested 15–84 d after transfer to the hydroponic system, depending on growth rates (Supplementary Data Table S1). Four species – (1) *Beta vulgaris* (Amaranthaceae; Caryophyllales), (2) *Helianthus annuus* (Asteraceae; Asterales), (3) *Hordeum vulgare* (Poaceae; Poales) and (4) *Sagina subulata* (Sw.) C.Presl (Caryophyllaceae; Caryophyllales) – were grown in gullies that were supplied by both tanks. Harvested plants were rinsed in deionized water and separated into shoots and roots. Shoots and roots were dried separately in paper bags at 70 °C for a minimum of 72 h to achieve a constant DW. Samples were milled to a fine powder using a ceramic ball mill (Retsch MM 200 or Retsch MM 301; Retsch, Haan, Germany) and accurately weighed powdered subsamples (~50 mg DW) were digested in nitric acid in closed vessels using a microwave digester (MARS Xpress, CEM Microwave Technology, Buckingham, UK) as described by White *et al.* (2012). Sodium concentrations in digested samples were measured using inductively coupled plasma mass spectrometry (ICP-MS; ELAN DRCe; PerkinElmer, Waltham, USA) as described by White *et al.* (2012). An externally certified reference material (1573a tomato leaf standard; National Institute of Standards and Technology, NIST, USA) was included as an internal control. Multiple replicates of individual genotypes were combined for ICP-MS analyses if insufficient dried sample was available. There was insufficient root dry matter of *Melandrium keiskei* (Miq.) Ohwi (Caryophyllaceae; Caryophyllales) to determine its sodium concentration. For this reason, data from *M. keiskei* were excluded from the results described below.

Eighteen Caryophyllales species, representing six Caryophyllales families, were grown in both the hydroponic experiment performed here and experiments described by White *et al.* (2017). These species were *Agrostemma githago* L., *Amaranthus caudatus* L., *Amaranthus cruentus* L., *Armeria maritima* (Mill.) Willd., *Atriplex hortensis* L., *Beta vulgaris*, *Carpobrotus edulis* (L.) N.E.Br., *Cerastium tomentosum* L., *Delosperma cooperi* (Hook.f.) L.Bolus, *Dorotheanthus bellidiformis* (Burm.f.) N.E.Br., *Limonium sinuatum* (L.) Mill., *Persicaria capitata* (Buch.-Ham. ex D.Don) H.Gross, *Phytolacca americana* L., *Plumbago auriculata* Lam., *Psylliostachys suworowi* (Regel) Roshkova, *Rheum palmatum* L., *Sagina subulata* and *Silene armeria* L. (Table 1).

Data analysis

Data analyses were conducted using R 3.4.3 (R Core Team, 2017) using the packages ape 5.0 (Paradis *et al.*, 2004), ggplot2 3.2.1 (Wickham, 2016), phangorn 2.3.1 (Schliep, 2011), phytools 0.6-44 (Revell, 2012) and rentrez 1.1.0 (Winter, 2017). Shoot sodium concentrations ($[Na]_{shoot}$) are expressed on a DW basis and variation is expressed as standard deviation of *n* observations unless indicated otherwise.

Block effects and differences between the two groups of four gullies supplied by each tank were tested by analysis of variance (ANOVA) using the 'aov' function from base R (R Core Team, 2017) and a linear model of the form $\log_e([Na]_{shoot}) \sim$ tank + block + genotype × organ. The tilde separates the response variable (left) from the explanatory variables (right) and × indicates a genotype × organ (i.e. shoot or root) interaction. The three genotypes barley (*Hordeum vulgare*; Poales), beetroot (*Beta vulgaris*; Caryophyllales) and sunflower (*Helianthus annuus*; Asterales), which were grown in both groups of four gullies, were included in this analysis. The fourth species grown in both groups of four gullies, *Sagina subulata* (Caryophyllales), was not included in this analysis as multiple shoot and root samples had to be combined to obtain enough material for ICP-MS analyses.

The trait of abnormally large $[Na]_{shoot}$ was defined by fitting log-normal distributions to the observed frequency distributions of $[Na]_{shoot}$ using the function 'rnorm' from base R. Log-normal distributions were compared with each other by conducting *t*-tests using the 't.test' function from base R. Species with marginal $[Na]_{shoot}$ to either of the log-normal distributions were assigned to a distribution using the 'pnorm' function from base R. Pearson's linear correlation coefficients

were determined and significance tests for correlations between $[Na]_{shoot}$ and root Na concentrations ($[Na]_{root}$) were conducted using the 'cor.test' function from base R. The 'density' function from base R was used for visualizing the empirical distributions of $[Na]_{shoot}$, $[Na]_{root}$ and $[Na]_{shoot}/[Na]_{root}$ of Caryophyllales species grown in the experiment (Fig. 1) based on kernel density estimates (KDEs).

The mean $[Na]_{shoot}$, $[Na]_{root}$ and $[Na]_{shoot}/[Na]_{root}$ of Caryophyllales families represented in the experiment were mapped to the matK phylogeny using the 'contMap' function of the phytools package (Revell, 2012).

To construct the matK phylogeny of the 13 Caryophyllales families represented in the experiment, amino acid sequences of the plastid gene *matK* were sourced using the NCBI protein database (Supplementary Data Table S2). Complete matK sequences were used for all genera, if possible, and partial matK sequences were sourced if no complete matK sequences were available. All amino acid sequences were obtained using the 'entrez_fetch' function from the rentrez package (Winter, 2017). All complete matK sequences were then aligned using MUSCLE 3.8.31 (Edgar, 2004). Unique partial matK sequences were then aligned iteratively against the alignment of complete matK sequences. A phylogenetic tree based on maximum likelihood was inferred using the alignment of both complete and partial sequences using the package phangorn (Schliep, 2011). The Bayesian information criterion (BIC), obtained using the 'modelTest' function of phangorn, suggested a JTT + G + I model. The topology of the phylogenetic tree was optimized using nearest neighbour interchanges (NNIs). The non-Caryophyllales species *Brassica oleracea* (Brassicales), *Helianthus annuus* (Asterales), *Hordeum vulgare* (Poales) and *Phlomis elliptica* Benth. (Lamiales) were used as an outgroup and were subsequently removed from the rooted tree of Caryophyllales families. Partial sequences of matK from

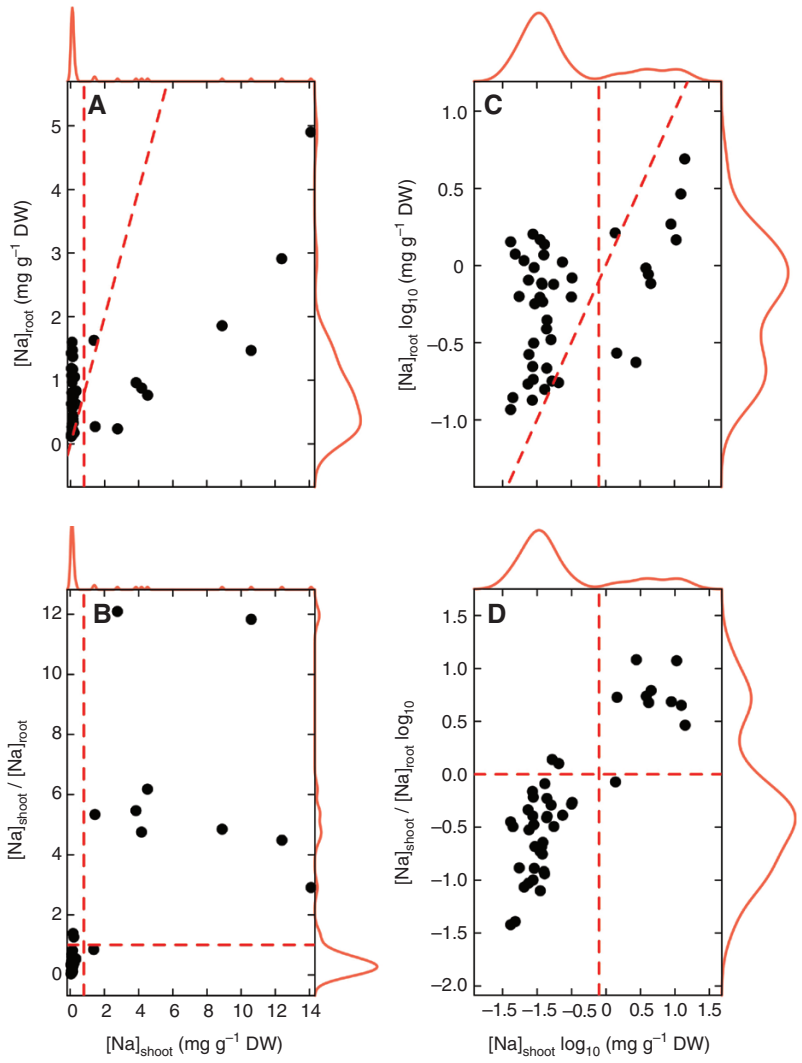

FIG. 1. (A) Mean $[Na]_{shoot}$ and $[Na]_{root}$ of 44 Caryophyllales species grown in non-saline solution, (B) mean $[Na]_{shoot}$ of the same species plotted against their respective $[Na]_{shoot}/[Na]_{root}$ quotients, (C) $\log_{10}$-transformed $[Na]_{shoot}$ and $[Na]_{root}$ and (D) $\log_{10}$-transformed $[Na]_{shoot}$ plotted against $[Na]_{shoot}/[Na]_{root}$ quotients. Vertical dashed red lines indicate $[Na]_{shoot} = 10^{-0.1}$ or $[Na]_{shoot}\log_{10} = -0.1$. Diagonal dashed lines and horizontal dashed lines indicate $[Na]_{shoot}/[Na]_{root}$ of unity. Red solid lines at the plot margins indicate empirical distribution functions based on kernel density estimates. Data for individual species can be found in Supplementary Data Table S1.

each genus clustered together, as did the sequences of matK for all genera within a Caryophyllales family.

Local regression analyses of the data sourced from the literature (Supplementary Data Table S3) were based on locally estimated scatterplot smoothing (LOESS) using the package ggplot2 (Wickham, 2016). The association between the accumulation of Na and the elements chlorine (Cl), S, nitrogen (N), phosphorus (P), potassium (K), Mg and Ca in shoots of Caryophyllales was tested by fitting linear models of the form $\log_e([\text{Element}]_{shoot}) \sim$ type for each element using the 'lm' function from base R (R Core Team, 2017). The tilde separates the response variable (left) from the explanatory variable (right). The data for this analysis were sourced from supporting information in Table S1 of Neugebauer *et al.* (2018). Caryophyllales species for which the element concentrations were determined were grouped into species with abnormally large Na concentrations (type 'hyper') and those with normal Na concentrations (type 'normal').

RESULTS

Sodium concentrations in shoots and roots of angiosperms

Sodium (Na) concentrations in shoots ($[Na]_{shoot}$) and roots ($[Na]_{root}$) were determined in 44 Caryophyllales species and four non-Caryophyllales species (Supplementary Data Table S1). No significant ($P < 0.05$) block effects or differences between the two groups of four gullies were observed for tissue Na concentrations of the three species present in both groups of four gullies, but differences in $[Na]_{shoot}$ among genotypes ($P < 0.001$), genotype × organ interactions ($P < 0.001$) and differences between roots and shoots ($P < 0.01$) were significant (Supplementary Data Table S4).

The $[Na]_{shoots}$ of all four non-Caryophyllales species (*Brassica oleracea*, *Helianthus annuus*, *Hordeum vulgare* and *Phlomis lychnitis*) were smaller than the mean $[Na]_{shoot}$ of the Caryophyllales species (1.550 ± 3.418 mg g^{-1} DW, $n = 44$ species; Supplementary Data Table S1) and consistent with the rank order of $[Na]_{shoot}$ of these species determined previously (White *et al.*, 2017). The $[Na]_{shoot}$ of all four non-Caryophyllales species fell within the range of $[Na]_{shoot}$ of Caryophyllales species that did not hyperaccumulate Na. The $[Na]_{root}$ of *Helianthus annuus*, *Hordeum vulgare* and *Phlomis lychnitis* were also smaller than the mean $[Na]_{root}$ of the Caryophyllales species studied (0.854 ± 0.856 mg g^{-1} DW, $n = 44$ species), but the $[Na]_{root}$ of *Brassica oleracea* was larger than the mean $[Na]_{root}$ of all Caryophyllales species. Nevertheless, the $[Na]_{root}$ of all non-Caryophyllales species fell within the range of $[Na]_{root}$ of the Caryophyllales species.

$[Na]_{shoot}$ of Caryophyllales species

The $[Na]_{shoot}$ of Caryophyllales species (Fig. 1) ranged from 0.04 ± 0.01 mg g^{-1} DW ($n = 2$ plants) in *Simmondsia chinensis* (Link) C.K. Schneid. (Simmondsiaceae) to 14.09 ± 1.2 mg g^{-1} DW ($n = 8$ plants) in *Carpobrotus edulis* (Aizoaceae). The distribution of $[Na]_{shoot}$ of 44 Caryophyllales species did not appear to fit a single normal distribution, nor a small set of normal distributions (Fig. 1A, B). However, the data did appear to fit a small number, possibly two, log-normal distributions (Fig. 1C, D). The first log-normal distribution, which had an estimated mean $[Na]_{shoot}$ of −0.975 ($\log_{10}$ mg g^{-1} DW), s.d. 0.225 ($\log_{10}$ mg g^{-1} DW), contained the 34 Caryophyllales species with the smallest $[Na]_{shoot}$. The second log-normal distribution, which had an estimated mean of 0.682 ($\log_{10}$ mg g^{-1} DW), s.d. 0.368 ($\log_{10}$ mg g^{-1} DW), contained the ten Caryophyllales species with the largest $[Na]_{shoot}$. The two distributions differed significantly ($P < 0.001$), suggesting two phenotypes. Species with $[Na]_{shoot}$ larger than ~0.8 mg g^{-1} DW ($\log_{10} = -0.1$) were more likely to belong to the second distribution and this threshold was used to assign Caryophyllales species a particular phenotype. The species with $[Na]_{shoot}$ closest to 0.8 mg g^{-1} DW were attributed a particular phenotype by testing the probabilities of these species belonging to either the first or the second distribution. *Psylliostachys suworowi* (Plumbaginaceae; 1.37 ± 0.58 mg g^{-1} DW, $n = 8$ plants) was attributed a phenotype of abnormally large $[Na]_{shoot}$ ($P = 0.069$) rather than a normal $[Na]_{shoot}$ ($P < 0.001$). *Cistanthe grandiflora* (Lindl.) Schltdl. (Portulacaceae; 0.32 ± 0.07 mg g^{-1} DW, $n = 8$ plants) was attributed a normal $[Na]_{shoot}$ ($P = 0.016$) rather than an abnormally large $[Na]_{shoot}$ ($P < 0.001$).

$[Na]_{root}$ of Caryophyllales species

The $[Na]_{root}$ among Caryophyllales species ranged from 0.12 ± 0.0001 mg g^{-1} DW ($n = 2$ plants) in *Simmondsia chinensis* (Simmondsiaceae) to 4.90 ± 0.7 mg g^{-1} DW ($n = 8$ plants) in *Carpobrotus edulis* (Aizoaceae). The distribution of $[Na]_{root}$ did not appear to fit a single normal distribution (Fig. 1A, B) or a single log-normal distribution (Fig. 1C, D). The distribution of $[Na]_{root}$ resembled a highly skewed normal distribution (Fig. 1A, B) or two overlapping log-normal distributions (Fig. 1C, D). The latter did not coincide with the two log-normal distributions observed for $[Na]_{shoot}$ (Fig. 1C, D). Overall, there was little correlation between $[Na]_{root}$ and $[Na]_{shoot}$ among Caryophyllales species (Fig. 1A, C). However, $[Na]_{root}$ and $[Na]_{shoot}$ were significantly correlated among the ten species with abnormally large $[Na]_{shoot}$ ($r = 0.83$, $P = 0.003$; Fig. 2B), but were completely uncorrelated among the 34 species with normal $[Na]_{shoot}$ ($r = -0.001$, $P = 0.996$; Fig. 2C). The variation of $[Na]_{shoot}$ of the latter 34 species was relatively narrow (0.121 ± 0.068 mg g^{-1} DW, $n = 34$ species), whereas their $[Na]_{root}$ (0.638 ± 0.447 mg g^{-1} DW) varied widely (Fig. 2C).

$[Na]_{shoot}/[Na]_{root}$ of Caryophyllales species

Quotients of $[Na]_{shoot}/[Na]_{root}$ ranged from 0.038 ± 0.0216 ($n = 2$ plants) in *Talinum paniculatum* (Jacq.) Gaertn. (Talinaceae) to 12.094 ± 2.551 ($n = 7$ plants) in *Atriplex halimus* L. (Amaranthaceae). The empirical distribution of $[Na]_{shoot}/[Na]_{root}$ from 44 Caryophyllales species did not fit a single normal distribution (Fig. 1B) or a single log-normal distribution (Fig. 1D).

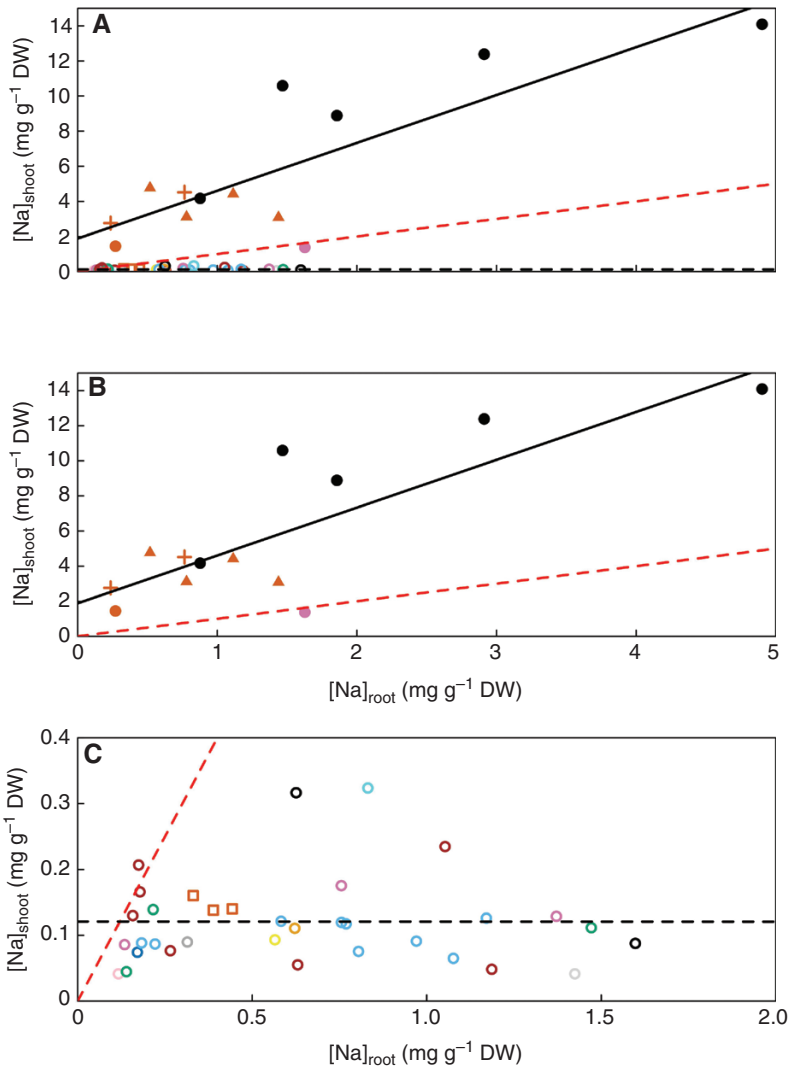

FIG. 2. Mean $[Na]_{shoot}$ and $[Na]_{root}$ of (A) 47 Caryophyllales genotypes grown in non-saline solution. Closed symbols and '+' indicate genotypes with abnormally large $[Na]_{shoot}$ and open symbols indicate genotypes with normal $[Na]_{shoot}$. Mean $[Na]_{shoot}/[Na]_{root}$ quotients of (B) the 13 Caryophyllales genotypes with abnormally large $[Na]_{shoot}$ and (C) the 34 Caryophyllales genotypes with normal $[Na]_{shoot}$. Colours indicate Aizoaceae (black), Amaranthaceae (red), Basellaceae (orange), Caryophyllaceae (light blue), Montiaceae (green), Nyctaginaceae (yellow), Petiveriaceae (dark blue), Phytolaccaceae (dark grey), Plumbaginaceae (dark pink), Polygonaceae (brown), Portulacaceae (cyan), Simmondsiaceae (light pink) and Talinaceae (light grey). Multiple genotypes of an Amaranthaceae species are indicated by circles, squares and '+'. The red dashed line indicates a $[Na]_{shoot}/[Na]_{root}$ quotient of unity. The black lines indicate the correlation between $[Na]_{shoot}$ and $[Na]_{root}$ among Caryophyllales with abnormally large $[Na]_{shoot}$ (solid, $r = 0.82$, $P < 0.001$, $n = 13$ genotypes) and normal $[Na]_{shoot}$ (dashed, $r = -0.001$, $P = 0.996$, $n = 34$). Data for individual species can be found in Supplementary Data Table S1.

Caryophyllales species with abnormally large $[Na]_{shoot}$ generally had $[Na]_{shoot}/[Na]_{root}$ quotients greater than unity, and Caryophyllales species with normal $[Na]_{shoot}$ generally had $[Na]_{shoot}/[Na]_{root}$ quotients less than unity (Fig. 1B, D). *Psylliostachys suworowi* ($[Na]_{shoot}$ = 1.375 ± 0.580, $[Na]_{shoot}/[Na]_{root}$ = 0.848 ± 0.346, n = 8 plants) was the only species with an abnormally large $[Na]_{shoot}$ and a $[Na]_{shoot}/[Na]_{root}$ quotient less than unity. The two Polygonaceae species *Emex australis* Steinh. ($[Na]_{shoot}$ = 0.207 ± 0.064, $[Na]_{shoot}/[Na]_{root}$ = 1.263 ± 0.470, n = 3 plants) and *Eriogonum arborescens* Greene ($[Na]_{shoot}$ = 0.166 ± 0.018, $[Na]_{shoot}/[Na]_{root}$ = 1.380 ± 0.817, n = 6 plants) did not accumulate abnormally large $[Na]_{shoot}$, but had mean $[Na]_{shoot}/[Na]_{root}$ quotients greater than unity. However, the $[Na]_{shoot}/[Na]_{root}$ quotients of these three species did not differ significantly ($P < 0.05$) from unity. Thus, the designation of abnormally large $[Na]_{shoot}$ coincided with a $[Na]_{shoot}/[Na]_{root}$ threshold of unity.

Some species in the three Caryophyllales families Aizoaceae, Amaranthaceae and Plumbaginaceae had abnormally large $[Na]_{shoot}$ (Fig. 2A, B), but no species in the remaining ten Caryophyllales families had abnormally large $[Na]_{shoot}$ (Supplementary Data Table S1). Some species from all of the 13 Caryophyllales families studied had normal $[Na]_{shoot}$ (Fig. 2C). Five of the seven Aizoaceae species studied had abnormally large $[Na]_{shoot}$ and these species generally had large $[Na]_{root}$ also. Four of the seven Amaranthaceae species studied had abnormally large $[Na]_{shoot}$: *Beta vulgaris* (all four genotypes: sugar beet, beetroot, sea beet, Swiss chard), both

Atriplex species (*A. halimus, A. hortensis*) and *Salicornia europaea* L. The $[Na]_{root}$ of these four Amaranthaceae species were within the range of $[Na]_{root}$ of Caryophyllales species exhibiting normal $[Na]_{shoot}$ (Fig. 2A). Thus, Amaranthaceae species with abnormally large $[Na]_{shoot}$ did not cluster together with Aizoaceae species exhibiting abnormally large $[Na]_{shoot}$ when $[Na]_{shoot}$ was plotted against $[Na]_{root}$ (Fig. 2B). One of the four Plumbaginaceae species studied, *Psylliostachys suworowi*, had an abnormally large $[Na]_{shoot}$. *Psylliostachys suworowi* also had a large $[Na]_{root}$. The three *Amaranthus* species (*A. caudatus*, *A. cruentus*, *A. tricolor*; Amaranthaceae) with normal $[Na]_{shoot}$ had similar $[Na]_{shoot}/[Na]_{root}$ quotients (Fig. 2C).

$[Na]_{shoot}$, $[Na]_{root}$ and $[Na]_{shoot}/[Na]_{root}$ quotient of Caryophyllales families

The mean $[Na]_{shoot}$, $[Na]_{root}$ and $[Na]_{shoot}/[Na]_{root}$ quotients were mapped to a matK phylogeny of the Caryophyllales families represented in this study (Fig. 3). Three of 13 Caryophyllales families studied had species with abnormally large $[Na]_{shoot}$, namely the Aizoaceae, Amaranthaceae and Plumbaginaceae (Supplementary Data Table S1). The mean $[Na]_{shoot}$ was also largest in these families (Fig. 3A). The Aizoaceae also had the largest mean $[Na]_{root}$ (Fig. 3B). However, although the Amaranthaceae and Plumbaginaceae had large mean $[Na]_{shoot}$ (Fig. 3A), they did not have large mean $[Na]_{root}$ (Fig. 3B),

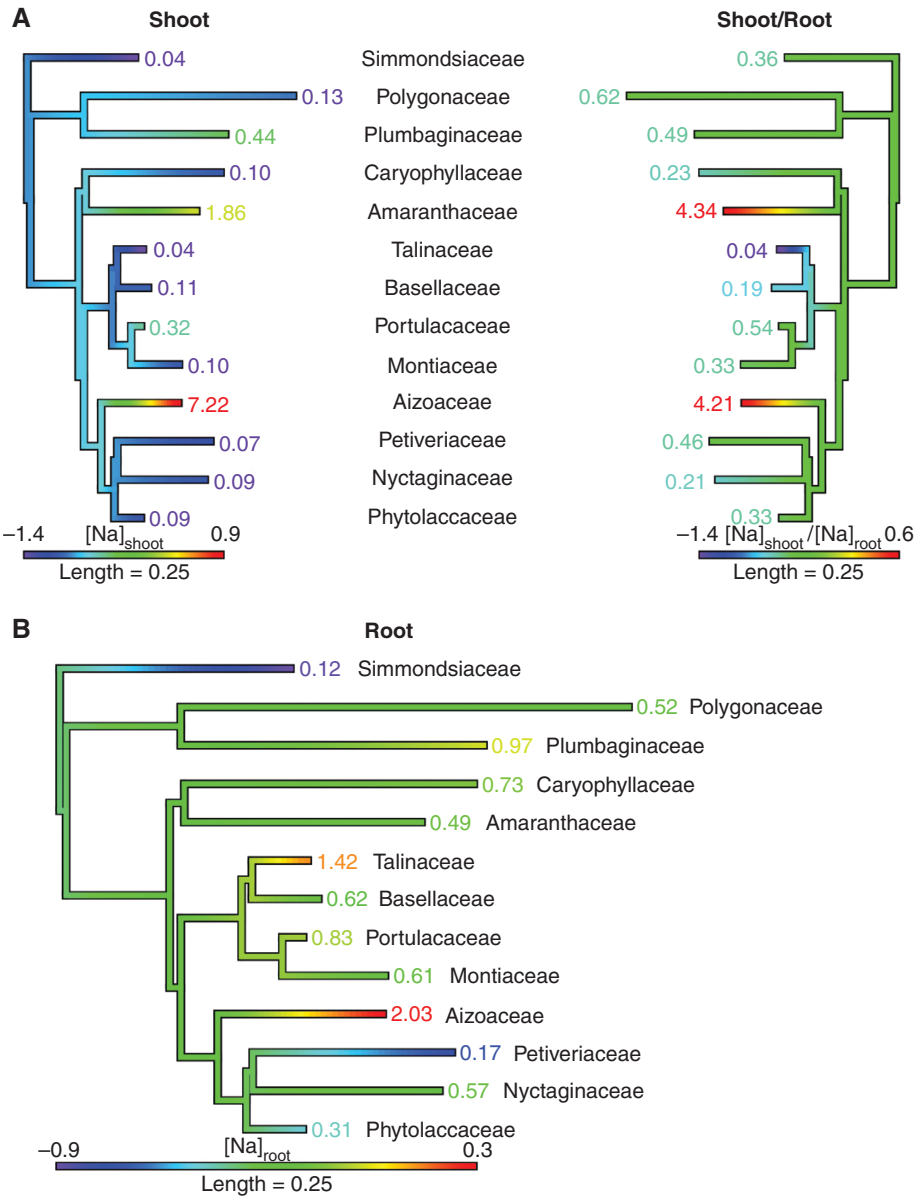

FIG. 3. Phylogenetic relationships among 13 Caryophyllales families based on matK. Mean $\log_{10}$-transformed $[Na]_{shoot}$ and $[Na]_{shoot}/[Na]_{root}$ quotients (A), and mean $[Na]_{root}$ (B) are mapped to the phylogeny using maximum likelihood interpolation. The relative lengths of the scale bars are the same for each phylogeny. Numbers at the tips indicate untransformed family mean values and their colours indicate the $\log_{10}$-transformed family mean values as shown in the colour scale bars.

and Talinaceae had small $[Na]_{shoot}$ but large $[Na]_{root}$ (Fig. 3B). Thus, the phylogenetic relationships among Caryophyllales families differed for $[Na]_{shoot}$ and $[Na]_{root}$. This is consistent with a lack of correlation between $[Na]_{shoot}$ and $[Na]_{root}$ among Caryophyllales species in general (Fig. 2C). The Aizoaceae and Amaranthaceae, the families with the most species with abnormally large $[Na]_{shoot}$ studied here, had the largest $[Na]_{shoot}/[Na]_{root}$ quotients of all the Caryophyllales families (Fig. 3A). The Aizoaceae and Amaranthaceae were the only families with a mean $[Na]_{shoot}$ >1 mg g^{-1} DW and a mean $[Na]_{shoot}/[Na]_{root}$ quotient greater than unity (Fig. 3A).

DISCUSSION

Sodium concentrations in shoots and roots of angiosperms

The mean $[Na]_{shoot}$ of the four non-Caryophyllales species (*Brassica oleracea*, *Helianthus annuus*, *Hordeum vulgare* and *Phlomis lychnitis*) was smaller than the mean $[Na]_{shoot}$ of the 44 Caryophyllales species studied (Supplementary Data Table S1) and the rank order of the four non-Caryophyllales species was consistent with the rank order of $[Na]_{shoot}$ of these species determined by White *et al.* (2017). The $[Na]_{shoot}$ of species can thus be compared between the two studies. In agreement with White *et al.* (2017), the $[Na]_{shoot}$ of all four non-Caryophyllales species was similar to the $[Na]_{shoot}$ of Caryophyllales species that did not hyperaccumulate $[Na]_{shoot}$. Root sodium concentrations ($[Na]_{root}$) of the four non-Caryophyllales species did not appear to differ from the $[Na]_{root}$ of Caryophyllales, indicating that the abnormally large $[Na]_{shoot}$ among some Caryophyllales did not require the accumulation of a large $[Na]_{root}$.

$[Na]_{shoot}$ in Caryophyllales

In agreement with White *et al.* (2017), $[Na]_{shoot}$ of the 44 Caryophyllales species grown in non-saline solution could be used to define two distinct $[Na]_{shoot}$ phenotypes (Fig. 1). The marginal $[Na]_{shoot}$ of *Cistanthe grandiflora* and *Psylliostachys suworowi*, with respect to the two $[Na]_{shoot}$ phenotypes, and their attribution of either phenotype were consistent with White *et al.* (2017). Four *Beta vulgaris* genotypes (sugar beet, beetroot, Swiss chard, sea beet), two *Atriplex* species (*Atriplex halimus*, *A. hortensis*) and three *Amaranthus* species (*Amaranthus caudatus*, *A. cruentus*, *A. tricolor* L.) had similar $[Na]_{shoot}$ (Fig. 2, Supplementary Data Table S1). Thus, there appeared to be little variation in $[Na]_{shoot}$ among genotypes of individual Amaranthaceae genera.

The threshold between the normal $[Na]_{shoot}$ and abnormally large $[Na]_{shoot}$ distributions differed between the hydroponic experiment presented here and the hydroponic experiments reported by White *et al.* (2017). Nevertheless, the evolutionary origins of the abnormally large $[Na]_{shoot}$ among Caryophyllales coincided (Figs 2 and 3). In addition, log-transformed $[Na]_{shoot}$ concentrations of the Caryophyllales species represented in the two studies were strongly correlated (Fig. 4; $r = 0.97$, $P < 0.001$, $n = 18$ species). The slope of the linear regression between $\log_{10}$-transformed $[Na]_{shoot}$ of Caryophyllales species represented in both studies was ~1, indicating a constant shift

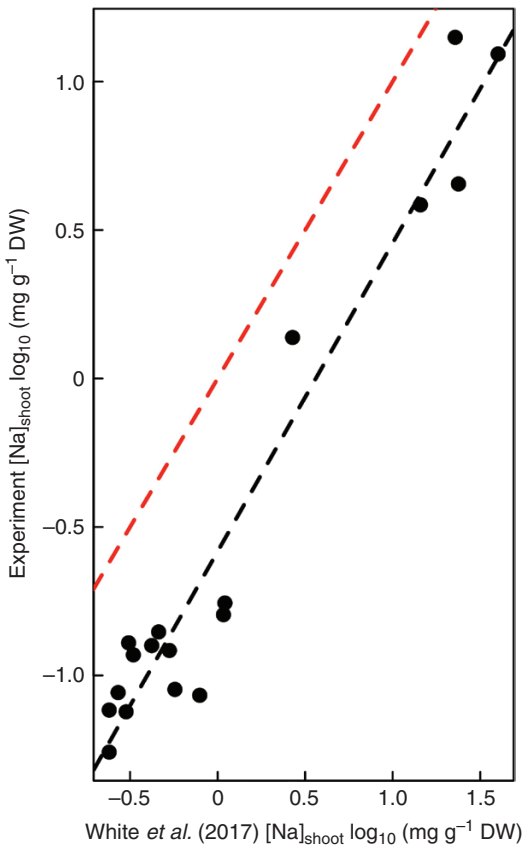

FIG. 4. $[Na]_{shoot}$ of Caryophyllales species represented in the present study and that of White *et al.* (2017). The linear regression (black) between the two datasets is $y = -0.582 + 1.039x$, with y and x indicating the $\log_{10}([Na]_{shoot})$ of the data presented by White *et al.* (2017) and the present study, respectively. The red line indicates $y = 0 + 1x$.

in observed $[Na]_{shoot}$. Therefore, the hypothesis (Hypothesis 1) that Caryophyllales species grown hydroponically in non-saline solution can be attributed a 'normal' or 'abnormally large' $[Na]_{shoot}$ phenotype proposed by White *et al.* (2017) could be confirmed. A non-saline nutrient solution, in which the Na reflects only Na contamination of used mineral salts, was used in both studies. A larger number of plants was grown in the hydroponic experiment presented here by extending the capacity of an existing hydroponic system by 33 % and by using the full capacity of the system. Thus, the differences in absolute $[Na]_{shoot}$ between studies might reflect changes in the Na available to plants (Glenn and O'Leary, 1984; Borer *et al.*, 2019). Log-normal $[Na]_{shoot}$ distributions can be used to define abnormally large $[Na]_{shoot}$ among Caryophyllales species grown in the same environment, but not across environments.

$[Na]_{root}$ in Caryophyllales

Sodium concentrations in roots of the 44 Caryophyllales species could not be used to define distinct $[Na]_{root}$ phenotypes and there was little correlation between $[Na]_{root}$ and $[Na]_{shoot}$ among Caryophyllales species (Fig. 1A). Thus, the evolution of abnormally large $[Na]_{shoot}$ among Caryophyllales did not require the evolution of abnormally large $[Na]_{root}$. However, $[Na]_{shoot}$ and

$[Na]_{root}$ were strongly correlated among hydroponically grown Caryophyllales species with abnormally large $[Na]_{shoot}$, but not among species with normal $[Na]_{shoot}$ (Figs 1 and 2C). The hypothesis (Hypothesis 2) that $[Na]_{shoot}$ is correlated with $[Na]_{root}$ among Caryophyllales grown in the same environment could thus be rejected. Sodium is transported with the transpiration stream via the xylem in plants (Broadley *et al.*, 2012) and accumulates in transpiring leaves. The wide range of $[Na]_{root}$ compared with the narrow range of $[Na]_{shoot}$ among Caryophyllales species with normal $[Na]_{shoot}$ suggests that some of the species studied restricted Na uptake into roots, whilst others restricted the translocation of Na from roots to shoots or actively removed Na from shoots either via the phloem or via salt extrusion mechanisms. Sodium appeared to be partitioned more readily from roots to shoots among Caryophyllales with abnormal $[Na]_{shoot}$.

$[Na]_{shoot}/[Na]_{root}$ in Caryophyllales species

The distribution of $[Na]_{shoot}/[Na]_{root}$ quotients from 44 Caryophyllales species could not be used to define distinct phenotypes (Fig. 1B, D). However, Caryophyllales species with abnormally large $[Na]_{shoot}$ generally had $[Na]_{shoot}/[Na]_{root}$ above unity, and Caryophyllales species with normal $[Na]_{shoot}$ generally had $[Na]_{shoot}/[Na]_{root}$ below unity (Figs 1 and 2). A $[Na]_{shoot}/[Na]_{root}$ quotient above unity suggests that Na is more readily partitioned to the shoot or that Na removal from the shoot is restricted. The empirical threshold for abnormally large $[Na]_{shoot}$ coincided with a $[Na]_{shoot}/[Na]_{root}$ threshold of unity. A shoot/root concentration quotient above unity has also been observed for plants that hyperaccumulate other mineral elements, such as Co, Ni, Mn and Zn (van der Ent *et al.*, 2013). Differences in environmental conditions, such as heavy metal toxicity or salinity, can affect the relative partitioning of Na between shoots and roots (Patel *et al.*, 1980). Thus, $[Na]_{shoot}/[Na]_{root}$ quotients might not be useful to define sodium hyperaccumulation in all environments. The relative partitioning of Na between shoots and roots is, however, likely to be more stable to environmental perturbation than $[Na]_{shoot}$. The hypothesis (Hypothesis 3) that the relative partitioning of [Na] can be used as an alternative criterion for defining Na hyperaccumulation in non-saline environments (cf. van der Ent *et al.*, 2013 for other elements) could be confirmed.

$[Na]_{shoot}$, $[Na]_{root}$ and $[Na]_{shoot}/[Na]_{root}$ of Caryophyllales families

The mean $[Na]_{shoot}$ of Caryophyllales families when mapped to a phylogeny (Fig. 3), suggests multiple evolutionary origins of the trait of abnormally large $[Na]_{shoot}$ in the Caryophyllales order. In agreement with White *et al.* (2017) and Ievinsh *et al.* (2021), the trait is unlikely to have evolved in an ancestor of the Amaranthaceae *sensu stricto*, but is common among species formerly classified Chenopodiaceae. In addition, abnormally large $[Na]_{shoot}$ is likely to have evolved in an ancestor of the Aizoaceae and during the evolution of the Plumbaginaceae. No species in the Montiaceae, Nyctaginaceae, Phytolaccaceae, Polygonaceae and Portulacaceae accumulated abnormally large $[Na]_{shoot}$ in the experiment reported here. White *et al.* (2017) reported that one of the two Portulacaceae species and two of the 20 Caryophyllaceae species they studied had abnormally large $[Na]_{shoot}$. Thus, the trait of abnormally large $[Na]_{shoot}$ is not likely to have evolved in ancestors of Montiaceae, Nyctaginaceae, Phytolaccaceae, Polygonaceae or Portulacaceae, although abnormally large $[Na]_{shoot}$ may have evolved within the Portulacaceae and Caryophyllaceae.

The mean $[Na]_{shoot}$ values of Caryophyllales families were not associated with the mean $[Na]_{root}$ of these families (Fig. 3). Thus, the evolution of a large $[Na]_{shoot}$ among Caryophyllales families did not require the evolution of a large $[Na]_{root}$. However, Caryophyllales families with large $[Na]_{shoot}$ generally had large $[Na]_{shoot}/[Na]_{root}$ quotients, suggesting that the accumulation of $[Na]_{shoot}$ is associated with a distinct partitioning of Na between shoots and roots among Caryophyllales with large $[Na]_{shoot}$.

$[Na]_{shoot}$, $[Na]_{root}$ and $[Na]_{shoot}/[Na]_{root}$ of Caryophyllales across environments

Data from the literature were sourced to validate the observations based on the hydroponic experiment described here for different environments (Supplementary Data Table S3). These data were collected from the papers cited by White *et al.* (2017), which included 29 publications that reported data on Na concentrations in either leaves or complete shoots ($[Na]_{top}$) and $[Na]_{root}$ on a DW basis. Experimental details, comprising the type of treatment (pot, hydroponics, agar, natural) and the Na concentration in the growth medium ($[Na]_{environment}$) were recorded where possible. Different growth conditions (e.g. different nutrient solutions) within a publication were considered individual studies. In total, 146 unique studies, representing a wide variety of experimental conditions (e.g. non-saline and saline conditions, mineral nutrient deficiency, heavy metal toxicity), were sourced from the literature. The dataset comprised $[Na]_{top}$ and $[Na]_{root}$ data on 39 Caryophyllales species representing 19 genera and eight families.

In agreement with the hydroponic study described here, $[Na]_{top}$ varied more than $[Na]_{root}$ across all Caryophyllales species and environments studied in the literature (Fig. 5A). The local regression curves indicated larger $[Na]_{top}$, $[Na]_{root}$ and $[Na]_{top}/[Na]_{root}$ quotients of Na hyperaccumulator species (n = 109 measurements) than non-hyperaccumulator species (n = 69 measurements). The mean $[Na]_{top}$ of Na hyperaccumulator species (94.10 ± 94.42 mg Na g^{-1} DW) was more than ten times larger than the mean $[Na]_{top}$ of non-hyperaccumulating species (9.29 ± 14.48 mg Na g^{-1} DW). In comparison, the mean $[Na]_{root}$ of Na hyperaccumulators (22.91 ± 24.52 mg Na g^{-1} DW) was about four times larger than the mean $[Na]_{root}$ of non-hyperaccumulating species (5.31 ± 8.42 mg Na g^{-1} DW). The mean $[Na]_{top}/[Na]_{root}$ quotient of Na hyperaccumulators (6.14 ± 7.73) was three times larger than the mean $[Na]_{top}/[Na]_{root}$ quotients of non-hyperaccumulating species (2.07 ± 1.35). Thus, many Caryophyllales species that do not hyperaccumulate Na in non-saline environments can have $[Na]_{shoot}/[Na]_{root}$ quotients above unity (Fig. 5B) in some environments. This shows that the

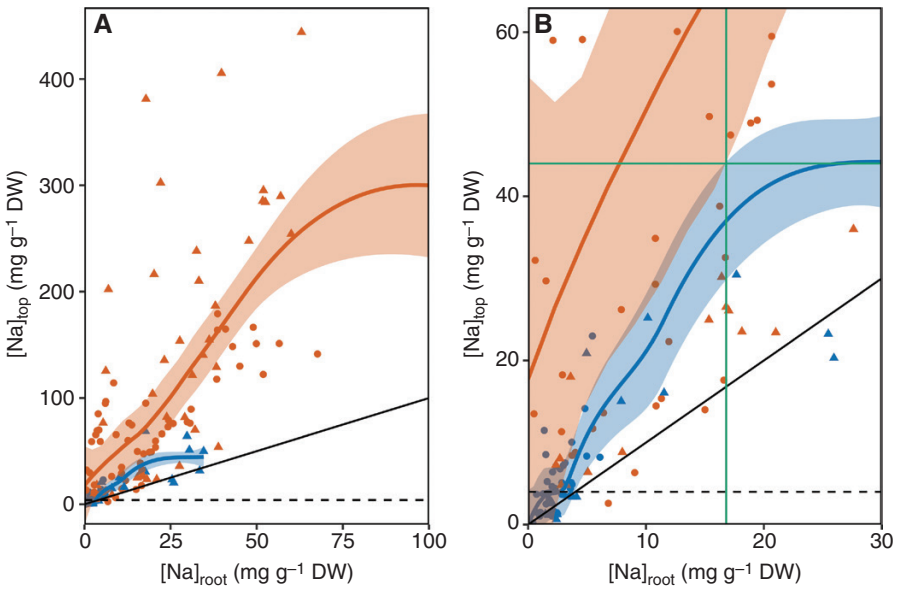

FIG. 5. Concentrations of sodium in whole shoots or leaves ($[Na]_{top}$) and in roots ($[Na]_{root}$) expressed on a DW basis sourced from the literature (Supplementary Data Table S3). The dataset contained 39 Caryophyllales species from eight families and 146 studies (A). Two measurements of *Suaeda monoica* (Amaranthaceae) with $[Na]_{root}$ >100 mg g^{-1} DW ($[Na]_{top}$ = 168.8 and 198.1 mg g^{-1} DW; $[Na]_{root}$ = 116.0 and 178.3 mg g^{-1} DW) are not shown. Colours indicate the unambiguous Na hyperaccumulators (red) and non-hyperaccumulators (blue) proposed by White *et al.* (2017). The fitted curves and 95 % confidence intervals (shaded) are based on LOESS and include the measurements that are not shown. Symbols indicate whole shoots (triangles) and leaves (circles). The solid black line indicates a $[Na]_{top}/[Na]_{root}$ quotient of unity and the dashed black line indicates $[Na]_{top}$ = 4 mg g^{-1} DW. A subset of the same data and regression is presented in panel (B), showing the approximate point of separation (green lines) between Na hyperaccumulators and non-hyperaccumulators at $[Na]_{top}$ = 44.0 mg g^{-1} DW and $[Na]_{root}$ = 16.8 mg g^{-1} DW.

$[Na]_{shoot}/[Na]_{root}$ threshold of unity did not hold true across all environments. However, the maximum $[Na]_{shoot}/[Na]_{root}$ quotients reached at extreme $[Na]_{environment}$ were much smaller in non-hyperaccumulator species than in Na hyperaccumulators.

The local regression curves of $[Na]_{top}$ against $[Na]_{environment}$ of Na hyperaccumulator species (n = 103 measurements) and non-hyperaccumulator species (n = 46 measurements) clearly separated irrespective of the salinity in the environment and despite the variation in $[Na]_{top}$ within both groups (Fig. 6A). The $[Na]_{top}$ of non-hyperaccumulator species ($[Na]_{top}$ = ~27 mg g^{-1} DW, $[Na]_{environment}$ = ~ 200 mM) plateaued at a much lower $[Na]_{environment}$ than the $[Na]_{top}$ of Na hyperaccumulator species ($[Na]_{top}$ = ~196 mg g^{-1} DW, $[Na]_{environment}$ = ~ 666 mM). Furthermore, the average $[Na]_{top}$ at the salinity threshold previously determined for $[Na]_{environment}$ (20 mM; White *et al.*, 2017) was 7.6 mg g^{-1} DW in non-hyperaccumulator species and 40.4 mg g^{-1} DW for Na hyperaccumulator species. This further indicates that the numerical $[Na]_{shoot}$ thresholds derived for individual non-saline hydroponic experiments are not universally valid for a range of salinities. Yet the intercept of non-hyperaccumulating Caryophyllales at $[Na]_{environment}$ = 0 mM ($[Na]_{top}$ = ~3.2 mg g^{-1} DW) was below the threshold of ~4 mg g^{-1} DW determined by White *et al.* (2017). In comparison, the intercept for Na hyperaccumulator species ($[Na]_{top}$ = ~25 mg g^{-1} DW) was much larger than that of non-hyperaccumulator species. The saturation of $[Na]_{shoot}$ among species grown in saline conditions might explain the low correlation between $[Na]_{shoot}$ and $[Na]_{environment}$ for plants growing in saline, coastal habitats found in the recent study by Ievinsh *et al.* (2021). Interestingly, the threshold for Na hyperaccumulation in shoots of species growing on these coastal habitats ($[Na]_{shoot}$ = 18–30 mg g^{-1} DW, $[Na]_{environment}$ ≥200 mS m^{-1}) coincided with the maximum $[Na]_{top}$ of Caryophyllales species that did not hyperaccumulate Na in their shoots observed here (Fig. 6A).

The local regressions of $[Na]_{root}$ against $[Na]_{environment}$ of Na hyperaccumulator species and non-hyperaccumulator species overlapped irrespective of $[Na]_{environment}$ (Fig. 6B). However, the average $[Na]_{root}$ of hyperaccumulator species was larger than that of non-hyperaccumulators. This agrees with the observations made in the hydroponic experiment described in this study and suggests that $[Na]_{root}$ alone is not sufficient to define the trait of Na hyperaccumulation in Caryophyllales.

The regression curves of $[Na]_{top}/[Na]_{root}$ against $[Na]_{environment}$ of Na hyperaccumulator species and non-hyperaccumulator species differed, irrespective of $[Na]_{environment}$ (Fig. 6C). The intercept of the regression of $[Na]_{top}/[Na]_{root}$ against $[Na]_{environment}$ ($[Na]_{environment}$ = 0 mM) was ~1.2/1 for non-hyperaccumulator species, and the 95 % confidence interval (CI) at the intercept included unity (Fig. 6D). In comparison, the intercept of the regression of $[Na]_{top}/[Na]_{root}$ against $[Na]_{environment}$ for Na hyperaccumulator species was 6.4/1 and the 95 % CI did not include unity. This is consistent with the observations made in our hydroponic experiment. The average $[Na]_{top}/[Na]_{root}$ quotients of Na hyperaccumulators and non-hyperaccumulators at $[Na]_{environment}$ = 20 mM were ~6.9/1 and 1.9/1, respectively. This suggests that $[Na]_{top}/[Na]_{root}$ quotients in Caryophyllales may increase with increasing $[Na]_{environment}$. The $[Na]_{top}/[Na]_{root}$ quotients of Na hyperaccumulator species and non-hyperaccumulator species plateaued at ~7.2/1 and 2.5/1, respectively. Thus, a $[Na]_{top}/[Na]_{root}$ threshold above unity might be more conservative for defining Na hyperaccumulation among Caryophyllales species across environments.

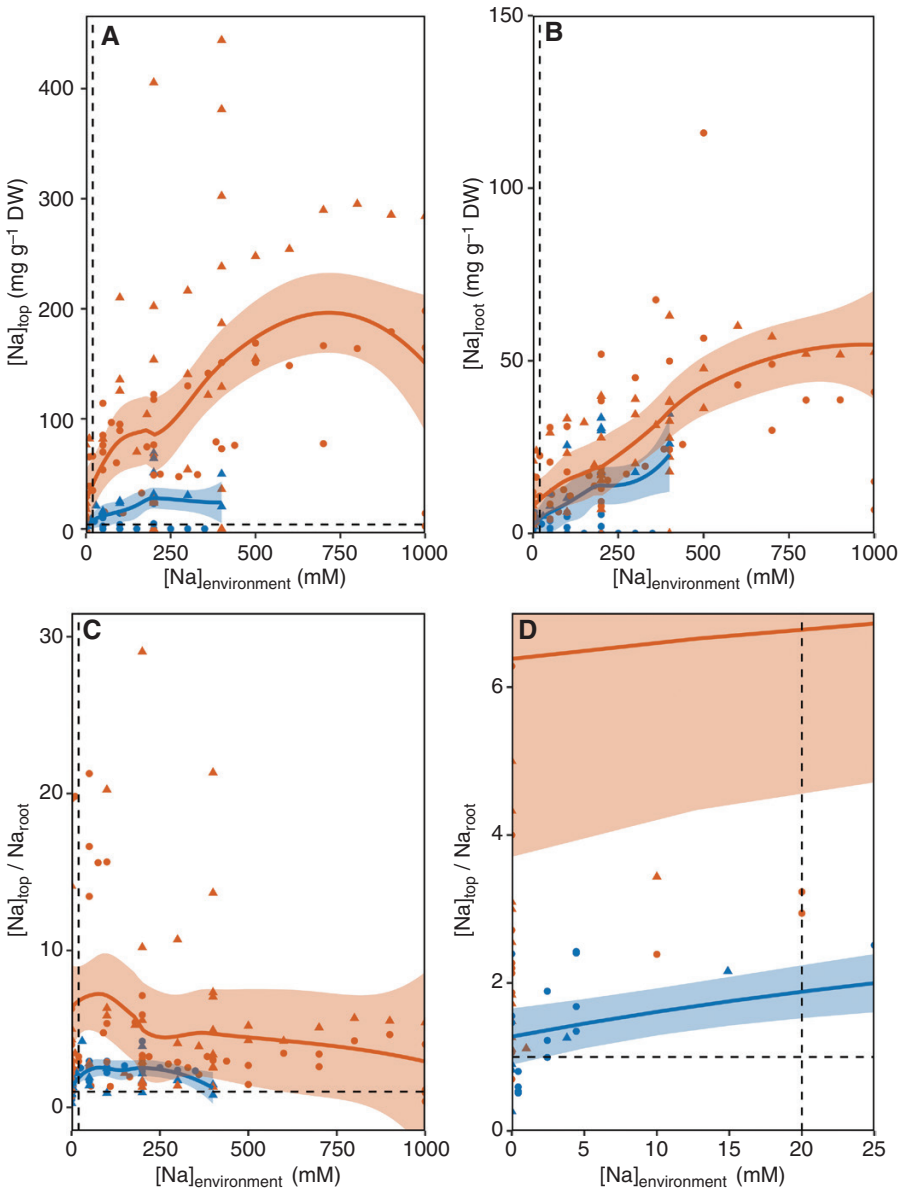

FIG. 6. Concentrations of sodium in (A) complete shoots or leaves ($[Na]_{top}$) and in (B) roots ($[Na]_{root}$) on a DW basis, and $[Na]_{top}/[Na]_{root}$ quotients (C, D) plotted against the Na concentration in the environment ($[Na]_{environment}$). Data are sourced from the literature (Supplementary Data Table S3) and represent 33 Caryophyllales species from seven families and 127 studies. One measurement of *Suaeda monoica* (Amaranthaceae) with $[Na]_{root}$ >150 mg g^{-1} DW ($[Na]_{top}$ = 198.1 mg g^{-1} DW; $[Na]_{root}$ = 178.3 mg g^{-1} DW) is not shown in (B) and one measurement of *Atriplex hymenelytra* (Amaranthaceae) with $[Na]_{top}/[Na]_{root}$ = 53.7 is not shown in (C). Colours indicate unambiguous hyperaccumulators (red) and non-hyperaccumulators (blue) identified by White *et al.* (2017). The fitted curves and 95 % confidence intervals (shaded) are based on LOESS and include the measurements that are not shown. Symbols indicate complete shoots (triangles) and leaves (circles). The vertical dashed lines indicate $[Na]_{environment}$ = 20 mM and the horizontal dashed lines indicate $[Na]_{top}$ = 4 mg g^{-1} DW and $[Na]_{top}/[Na]_{root}$ of unity.

It is worth highlighting that the reported values of $[Na]_{environment}$ in individual studies may not be precise. Thus, even $[Na]_{environment}$ of 0 mM may not be exactly zero due to Na contamination, as shown by our own hydroponic experiments. Yet the observations from hydroponic experiments using non-saline nutrient solution described here and previously (White *et al.*, 2017) are generally in agreement with the literature. Thus, the hypothesis (Hypothesis 4) that observations made for Caryophyllales grown hydroponically under non-saline conditions can be generalized across environments was not rejected. However, the exact numerical thresholds inferred in individual studies may differ because the uptake and partitioning of Na by Caryophyllales can change as a consequence of different mineral nutrition and with different salinity, in particular. Mineral nutrient deficiencies and toxicities of mineral elements are common in both natural and agricultural environments (White *et al.*, 2013). Evidence was provided here that differences in $[Na]_{shoot}$ and $[Na]_{shoot}/[Na]_{root}$ between Caryophyllales species that hyperaccumulate Na and those that do not can be observed across environments (Figs 4–6). Using $[Na]_{shoot}/[Na]_{root}$ quotients in addition to $[Na]_{shoot}$ to identify Na hyperaccumulator species in the Caryophyllales may be preferable when studying

Caryophyllales grown in their natural environments, without controlled mineral nutrition. Provided clean roots can be obtained from the substrate, this may enable the study of the evolution of Na hyperaccumulation among Caryophyllales that are difficult or slow to grow in controlled environments, such as Cactaceae. Abnormally large $[Na]_{shoot}$ has previously been reported for some Cactaceae grown in non-saline environments (White *et al.*, 2017). No Cactaceae species were grown hydroponically in the experiment reported here due to their slow growth rates. Future studies could test the prevalence of Na hyperaccumulation among Cactaceae and other Caryophyllales that are difficult to grow hydroponically by characterizing their $[Na]_{shoot}$ as well as their $[Na]_{shoot}/[Na]_{root}$ quotients.

The genetic mechanisms controlling the hyperaccumulation of Na in non-saline conditions remain unknown. Sodium homeostasis in plants is likely to be controlled by many genes (Zhang *et al.*, 2017). Nevertheless, experiments in the model plant *Arabidopsis thaliana* indicate that *AtHKT1*- and *AtSOS1*-like genes could be candidate genes contributing to the trait of Na hyperaccumulation. *HKT1* is expressed in both roots and leaves in arabidopsis and *athkt1* knockout mutants have smaller $[Na]_{root}$ but larger $[Na]_{shoot}$ and thus larger $[Na]_{shoot}/[Na]_{root}$ quotients (Mäser *et al.*, 2002). Conversely, greater expression of *AtHKT1;1* in roots may cause smaller $[Na]_{shoot}$ due to its proposed role in retrieving Na from the xylem sap (Jha *et al.*, 2010). *HKT1*-type genes are also involved in Na homeostasis in other angiosperms, including tomato (Jaime-Pérez *et al.*, 2017), wheat (James *et al.*, 2011) and the halophytic grass *Puccinellia tenuiflora* (Griseb.) Scribn. & Merr. (Zhang *et al.*, 2017). The ability to hyperaccumulate Na in shoots would imply greater tolerance for Na in shoot tissues, although this has not been demonstrated formally here.

Associations between Na hyperaccumulation and the accumulation of other elements

The accumulation of Na^+ in shoots requires the accumulation of negatively charged counterions, such as Cl^-, sulphate, nitrate, phosphate or organic anions. However, the distinct phenotype of abnormally large $[Na]_{shoot}$ in Caryophyllales species studied here and by White *et al.* (2017) did not appear to be associated with abnormally large shoot concentrations of Cl, S, N or P (Neugebauer *et al.*, 2018; Table 2). Similarly, the accumulation of abnormally large $[Na]_{shoot}$ did not appear to be associated with abnormally large concentrations of K, Ca or Mg in shoots (Neugebauer *et al.*, 2018; Table 2). Species in the Caryophyllales often have greater shoot/root concentration quotients of P, K, Mg, Fe, Mn, Cu, Zn and Ni than other angiosperms (Neugebauer, 2019). However, although these elements showed variation in their shoot/root concentration quotients among Caryophyllales species, this was not associated with large concentrations of these elements in the shoot (Neugebauer, 2019).

Conclusions

Two distinct Caryophyllales $[Na]_{shoot}$ phenotypes, 'normal $[Na]_{shoot}$' and 'abnormally large $[Na]_{shoot}$', can be defined.

Table 2. *Mean log_e-transformed element concentrations (mg g^{-1} dry weight) and 95 % confidence intervals (95 % CI) in shoots of Caryophyllales species reported by Neugebauer et al. (2018). Species for which the elements Na, Cl, S, N, P, K Mg and C were measured were grouped into those with abnormally large Na concentrations (hyper) and those with normal Na concentrations (normal), as identified by White et al. (2017)*

Element	Type	Mean (95 % CI)	Species
Na	Normal	−0.87 (−1.08, −0.66)	49
	Hyper	2.78 (2.36, 3.21)	12
Cl	Normal	1.48 (1.35, 1.61)	22
	Hyper	1.37 (1.14, 1.60)	7
S	Normal	1.29 (1.18, 1.39)	22
	Hyper	1.44 (1.25, 1.62)	7
N	Normal	3.96 (3.89, 4.03)	38
	Hyper	3.87 (3.73, 4.01)	9
P	Normal	2.14 (2.01, 2.28)	49
	Hyper	2.22 (1.94, 2.49)	12
K	Normal	3.68 (3.57, 3.79)	49
	Hyper	3.90 (3.68, 4.12)	12
Mg	Normal	1.72 (1.58, 1.85)	49
	Hyper	1.96 (1.69, 2.23)	12
Ca	Normal	2.25 (2.11, 2.39)	49
	Hyper	2.19 (1.89, 2.48)	12

Species that exhibited normal $[Na]_{shoot}$ or abnormally large $[Na]_{shoot}$ in the experiment presented here also exhibited normal $[Na]_{shoot}$, or abnormally large $[Na]_{shoot}$ in the experiment presented by White *et al.* (2017). However, the $[Na]_{shoot}$ threshold defining Na hyperaccumulation differed between the two studies. This demonstrates that a numerical threshold for defining abnormally large $[Na]_{shoot}$ only applies to species grown under the same conditions. The $[Na]_{shoot}$ of Caryophyllales species was not correlated with their $[Na]_{root}$ and the evolution of abnormally large $[Na]_{shoot}$ did not appear to require the evolution of a large $[Na]_{root}$. However, species with abnormally large $[Na]_{shoot}$ generally have $[Na]_{shoot}/[Na]_{root}$ quotients greater than unity when grown under non-saline conditions and $[Na]_{shoot}/[Na]_{root}$ quotients can thus be used as an additional measure to define species that hyperaccumulate Na. The prevalence of Na hyperaccumulation in Caryophyllales families that are difficult to grow under controlled conditions, such as Cactaceae, remains unknown but may be explored using $[Na]_{shoot}/[Na]_{root}$ to confirm Na hyperaccumulation.

SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following. Table S1: taxonomy of the 52 angiosperm genotypes grown hydroponically in non-saline solution, their allocated shoot sodium phenotype, number of days grown hydroponically, shoot and root fresh and dry weights, and their shoot and root Na concentrations. Table S2: complete and partial matK sequences used for inferring the phylogeny of the 13 Caryophyllales families represented by 44 Caryophyllales species and matK sequences of the four non-Caryophyllales species used for rooting the phylogenetic tree. Table S3: sodium concentrations in tissues of Caryophyllales species sourced from the literature. Table S4: analysis of variance table for

sodium concentrations in shoots and roots of three genotypes grown in two groups of four gullies, each supplied by nutrient solution from a different tank and both divided into two blocks.

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