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Research

Adaptation to coastal soils through pleiotropic boosting of ion and stress hormone concentrations in wild *Arabidopsis thaliana*

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Summary

- Local adaptation in coastal areas is driven chiefly by tolerance to salinity stress. To survive high salinity, plants have evolved mechanisms to specifically tolerate sodium. However, the pathways that mediate adaptive changes in these conditions reach well beyond Na^+.
- Here we perform a high-resolution genetic, ionomic, and functional study of the natural variation in *Molybdenum transporter 1* (*MOT1*) associated with coastal *Arabidopsis thaliana* accessions. We quantify the fitness benefits of a specific deletion-harbouring allele ($MOT1^{DEL}$) present in coastal habitats that is associated with lower transcript expression and molybdenum accumulation.
- Analysis of the leaf ionome revealed that $MOT1^{DEL}$ plants accumulate more copper (Cu) and less sodium (Na^+) than plants with the noncoastal *MOT1* allele, revealing a complex interdependence in homeostasis of these three elements. Our results indicate that under salinity stress, reduced *MOT1* function limits leaf Na^+ accumulation through abscisic acid (ABA) signalling. Enhanced ABA biosynthesis requires Cu. This demand is met in Cu deficient coastal soils through $MOT1^{DEL}$ increasing the expression of *SPL7* and the copper transport protein *COPT6*.
- $MOT1^{DEL}$ is able to deliver a pleiotropic suite of phenotypes that enhance salinity tolerance in coastal soils deficient in Cu. This is achieved by inducing ABA biosynthesis and promoting reduced uptake or better compartmentalization of Na^+, leading to coastal adaptation.

Introduction

Our understanding of the genomic basis of adaptive variation is increasing, but there is still very little known about the pleiotropic consequences of adaptive variation. Powerful models of plant adaptation to environmental variation in mineral nutrient and trace elements offer promising starting points from which to improve our understanding of these pleiotropic consequences, which arise from the interconnectedness of ion homeostasis networks (Busoms *et al.*, 2015, 2018; Terés *et al.*, 2019). In particular, ionomic approaches enable global contrasting of mineral nutrient and trace element accumulation between species and environments, allowing broad insight into the consequences of adaptive variation at particular alleles. Combined with genome-wide association (GWA) studies, ionomics has allowed a far-reaching assessment of adaptive, naturally evolved changes in mineral nutrient uptake and storage (Huang & Salt, 2016).

Using such approaches, Baxter *et al.* (2008) identified *Molybdenum Transporter 1* (*MOT1*) as the causal gene driving reduced shoot molybdenum (Mo) in two accessions of *Arabidopsis thaliana* (Van-0 and Ler-0). Forsberg *et al.* (2015) showed that variation in shoot Mo content across 283 Arabidopsis accessions is controlled by variation in *MOT1*, and at least some of this variation may be adaptive to soil Mo concentrations (Poormohammad Kiani *et al.*, 2012). *MOT1* belongs to the sulphate transporter superfamily, is localized to mitochondria, and regulates whole-plant Mo homeostasis in both Arabidopsis (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008) and rice (Huang *et al.*, 2019). By dissecting the variance heterogeneity of *MOT1* in detail, Forsberg *et al.* (2015) characterized two major polymorphisms in *MOT1*: a deletion in the *MOT1* promoter ($MOT1^{DEL}$) and a duplication inside of a previous transposable element ($MOT1^{DUP}$). The duplication increases the expression of *MOT1* and results in higher accumulation of Mo in shoots; the deletion reduces expression of the *MOT1* gene and causes a reduction in whole-plant Mo content.

We have identified plants harbouring the reference-like *MOT1* allele (Col-0-like; $MOT1^{C}$) and plants with the two structural variants, $MOT1^{DEL}$ and $MOT1^{DUP}$ in numerous natural demes of Arabidopsis in the north-east of Spain (Busoms *et al.*, 2015), allowing a closer look at the mechanism underlying these observations. Interestingly, the $MOT1^{DEL}$ allele occurs primarily in coastal environments, and based on this distribution we suggest that *MOT1* loss-of-function may control tolerance to elevated

soil salinity at the coast. Several studies have shown that the application of Mo and copper (Cu) to soil increases fitness under salt stress, reducing sodium (Na^+) content and increasing both abscisic acid (ABA) biosynthesis and the ability of plants to take in essential macronutrients such as potassium (K^+) (e.g. Eskandari & Mozaffari, 2014; Wu *et al.*, 2017). Forsberg *et al.* (2015) detected an association with variation in leaf Mo, independent of *MOT1*, *c.* 600 kb upstream of *MOT1*. Using a T-DNA knockout allele, they proposed *Copper Transporter 6 (COPT6)* as a promising candidate gene for this association, suggesting a concrete genetic link between Mo and Cu homeostasis.

A further connection between Cu and Mo homeostasis is given by the fact that Cu is essential for ABA biosynthesis, because Cu is needed for the synthesis of the molybdenum cofactor Moco (Schwarz & Mendel, 2006). Abscisic acid plays a key role in plant responses to abiotic stress such as drought and salinity (Zhang *et al.*, 2006). Under stress conditions, tolerant plants accumulate ABA, inducing stress-responsive genes. The main site of Na^+ toxicity is usually the leaf blade; therefore, the activation of genes promoting the compartmentalization of Na^+ before it reaches the leaves is crucial for salinity tolerance (Munns & Tester, 2008). We hypothesized that $MOT1^{DEL}$ has a role in local adaptation to coastal saline habitats by enhancing *COPT6* expression, Cu uptake, Moco and ABA biosynthesis, resulting in less leaf Na^+ accumulation, thus allowing salinity stress resilience. To test this, we performed high-resolution, multiyear experiments in the field and under lab-controlled conditions, focusing on the complex interplay between Mo-Cu-Na^+ and their transporters. This set of laboratory and field-based experiments has led us to conclude that the naturally occurring $MOT1^{DEL}$ allele has an adaptive advantage in coastal conditions, providing enhanced salinity tolerance (NaCl) through the pleiotropic enhancement of both Cu and ABA concentrations.

Materials and Methods

Collection of plant and soil material

Thirty-six demes of *Arabidopsis thaliana* were selected in Catalonia (north-east Spain). A 'deme' is defined as a small group of Arabidopsis plants growing in relatively homogeneous ecological conditions and separated from other groups by at least 35 m. Each year (from 2013 to 2015) at each site, samples were collected such that half of each collected plant was taken for ionomic analysis and the other half was used to extract DNA for *MOT1* genotyping. Seeds of each individual were collected directly in the field and stored in packets over silica gel in a sealed box until they were used. For the soil elemental composition analysis, we collected three soil samples (*c.* 50 g of soil from the first 10 cm depth) at each site during the first wk of May of each year. Soil samples from the coastal and inland field sites were collected twice per month during Arabidopsis reciprocal transplants (from February to June of 2013, 2014 and 2015). Soil was air-dried under laboratory conditions, passed through a 2-mm sieve, and stored dry.

Soil and leaf elemental analysis

For soil analysis, we performed three independent soil analyses per site. To characterize the elemental composition of soil, analyses were performed on the 2-mm fraction samples following the extraction method described in Busoms *et al.* (2018). Plants from the field or the laboratory were sampled by removing 2–3 leaves (1–5 mg dry weight) and washed with ultrapure (18 MΩ) water before being placed in Pyrex digestion tubes. The digestion method and calibration are described in Busoms *et al.* (2018).

Single nucleotide polymorphism (SNP) genotyping and sequencing of *MOT1*

DNA from leaf material was extracted following the method detailed in Supporting Information Methods S1. An SSR marker (Simple Sequence Repeat) was developed based on the deletion in the promoter of *MOT1* in Van-0 plants (CS1584) (Baxter *et al.*, 2008) with forward primer 5′-CTCCGGTTATCGC GTTGTAT-3′ and reverse primer 5′-ACTGTCGCCATCA AGGTTTT-3′. We visualized, aligned, and blasted sequences from the NCBI database with Geneious v.10.0.9 (Kearse *et al.*, 2012).

Field-based reciprocal common garden experiments

After genotyping plants from 2012 and 2013 collections, we selected two demes containing plants with the $MOT1^C$ allele and plants with the $MOT1^{DEL}$ allele named T9 and LLO2. In March 2014 and March 2015, 100 seeds of each deme/*MOT1* variant ($T9^C$, $T9^{DEL}$; $LLO2^C$, $LLO2^{DEL}$) were sown at Blanes coastal field (lat. 41°40′37.64″, long. 2°48′3,86″) and at Santa Coloma de Farners inland field (lat.41°50′41.04″, long. 2°40′36.13″) into 16 × 16 cm squares. We studied the fitness of 10 plants for each deme/*MOT1* variant at each site, and the other 10 plants were harvested in April 2014 and April 2015 to analyse the leaf ionome. Rosette diameters were measured every wk for 2 months and the number of siliques was counted at maturity. Only seeds containing siliques were considered. The 2013 and 2014 reciprocal transplants are described in Busoms *et al.* (2015). Data were reanalysed, classifying the plants as $MOT1^{DEL}$ (shoot Mo^{2+} content < 3.5 μg g^{-1}); $MOT1^{DUP}$ (shoot Mo content > 9 μg g^{-1}) or $MOT1^C$ (Dataset S1).

Salinity tolerance assays

Seeds of the *mot1-2* (SALK 069683) and *copt6-1* (SALK 083438) T-DNA insertion alleles and *aba2-1* (CS 156) were obtained from the Arabidopsis Biological Resource Center (Alonso *et al.*, 2003). The same demes selected for the field common garden experiment, together with Col-0 and the *mot1-2* mutant plants, were used to perform irrigation, hydroponics and salt spray experiments with NaCl to test salinity tolerance and analyse the leaf ionome (procedure and growth conditions specified in Methods S2, S3).

Genotype data of the 1135 Arabidopsis strains were downloaded from http://1001genomes.org/ (1135_snp-short-indel.vcf.gz). *MOT1* allele type classification and normalized leaf values of 18 ions for the 1135 strains cultivated in common soil under control conditions can be found in Datasets S2, S3. Moreover, a worldwide set of 306 natural accessions was phenotyped for leaf Na^+ content. All the accessions are listed in Dataset S4 and *MOT1* type is indicated. The experimental design is detailed in Methods S4.

For the complementation study, we quantified the shoot Mo, Na^+ and Cu content (μg g^{-1} dry weight) of six plants of each type: Col-0, *mot1-2*, *copt6-1*, $T9^C$, $T9^{DEL}$, $LLO2^C$ and $LLO2^{DEL}$ and F1s from crosses between $T9^C \times T9^{DEL}$, $LLO2^{DEL} \times LLO2^C$ and $T9^{DEL} \times LLO2^{DEL}$ cultivated in common soil irrigated with half-strength Hoagland solution (pH 6.0) with 50 mM NaCl for 4 wk.

Expression (quantitative real-time polymerase chain reaction (qRT-PCR)) analysis

RNA from leaf and root material was extracted following the method described in Methods S1. Primers used for *MOT1* (At2g25680), *COPT6* (At2g26975), *SPL7* (At5g18830), *CNX6* (At2g43760), *CNX1* (At5g20990) and *ABA3* (At1g16540) transcript quantification are detailed in Table S1. For normalization across samples, the expression of the *ACTIN 1* gene (At2g37620) was analysed. For each sample, the average value from triplicate real-time PCRs was used to estimate transcript abundance. Data were analysed using the SDS v.1.0 software (Applied Biosystems 7900HT Fast Real-Time PCR System, Thermo Fisher Scientific - ES; https://www.thermofisher.com/es/es/home/brands/applied-biosystems.html). Threshold cycle (Ct) values were determined based on efficiency of amplification. The mean Ct values were normalized against *Actin1* gene and Ct values, calculated as (Ct_{Gene}–Ct_{Actin1}). The root and leaf expression of *MOT1* was calculated using the $2^{-\Delta Ct}$ method. The relative expression of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (NaCl treatment – Control).

Abscisic acid quantification

Abscisic acid concentration (nmol l^{-1}) was quantified by indirect enzyme-linked assay (ELISA) using a Phytodetek ABA Test Kit (Agdia, Elkhart, IN, USA) on three plants per treatment from T9 (Mix), T1 ($MOT1^{DEL}$) and PA10 ($MOT1^C$) demes, along with Col-0 and *mot1-2, copt6-1* and *aba2-1* mutants cultivated in common soil irrigated with 0 and 50 mM of NaCl for 2 wk.

Statistical analysis

For the hierarchical clustering, we generated a progressive alignment of 262 SNPs for the *MOT1* gene tree and 37 574 SNPs for the whole-genome tree from 74 plants from Busoms *et al.* (2018). Pairwise genetic distance between individuals and between demes was calculated using the Maximum Likelihood statistical method and the Jukes–Cantor substitution model using JMP v.13.0 (SAS, 2016). Mean-standardized values (−1< value >1) of elemental contents of soil and leaf material were used to represent the radar plots and compare *MOT1* allelic variants (Dataset S2). One-way or multivariate ANOVA was used to test for significant differences between means of fitness, elemental contents of soil and leaf material, gene expression and geostatistical variables. To test for correlations between two variables, a bivariate fit was applied. To perform multiple comparisons of group means we used Tukey's HSD (Dataset S5) in SAS Software JMP v.16.0 (https://www.jmp.com/es_es/home.html, 2016).

Results and discussion

Previous work has provided evidence that Arabidopsis populations from north-east Spain harbour substantial genetic variability and adaptive variation to elevated salinity in coastal soils at high geographic and genomic resolution (Busoms *et al.*, 2015, 2018). Here we build on these works, focussing on novel structural variation in *Molybdenum Transporter MOT1* and the adaptive consequences of this variation.

MOT1 allelic variation and distribution

Genome-wide association studies indicate that the *MOT1* locus exhibits contrasting effects on leaf Mo accumulation through different natural alleles (Forsbert *et al.*, 2015; Baxter *et al.*, 2008). Tomatsu *et al.* (2007) and Baxter *et al.* (2008) first detected a 53 bp deletion on the *MOT1* promoter associated with reduced *MOT1* expression and decreased shoot Mo content ($MOT1^{DEL}$). Forsberg *et al.* (2015) also found independent polymorphisms which were associated with increased *MOT1* expression and elevated shoot Mo concentration: two forms of a 330-bp-long duplication in a transposable element sequence, AT2TE47050 ($MOT1^{DUP}$); and two SNP markers, one located *c.* 25 kb downstream ($MOT1\text{-}SNP_1$) and another located *c.* 600 kb upstream of the *MOT1* coding locus ($MOT1\text{-}SNP_2$).

To determine which *MOT1* alleles are present in wild populations of Arabidopsis adapted to high soil salinity and naturally heterogenous soils on the north-east coast of Spain (Busoms *et al.*, 2015, 2018), we performed a high-resolution survey over 3 consecutive years (2013–2015). We collected, genotyped and quantified shoot Mo content from 36 demes (small groups of plants growing in relatively homogeneous ecological conditions; see the sub-section 'Collection of plant and soil material', above) in a region *c.* 60 km across (Fig. 1a). This yielded 25 demes containing only plants with the *MOT1* Col-0 reference-like allele $MOT1^C$ (Fig. 1a: black dots, coastal; grey dots, inland), while the rest of the demes harboured either mixtures of the other alleles or only $MOT1^{DEL}$ (Fig. 1a: green dots). Additionally, there were three demes located in the north of the area that contained mixtures of plants with either $MOT1^C$ or $MOT1^{DUP}$ alleles (Fig. 1a: red/black dots). The overall distribution of *MOT1* alleles was nonrandom across the study area: while we did not detect a correlation with soil Mo concentrations ($r^2 = 0.087$), we detected a significant association between the presence of $MOT1^{DEL}$ and the distance to the sea (ChiSq = 324,83; $P < 0.001$), with the demes containing only $MOT1^{DEL}$ allele being close to the coast (Fig. 1b), suggesting a potential adaptive value of this allele in coastal environments. Moreover, we did not detect $MOT1^{DEL}$

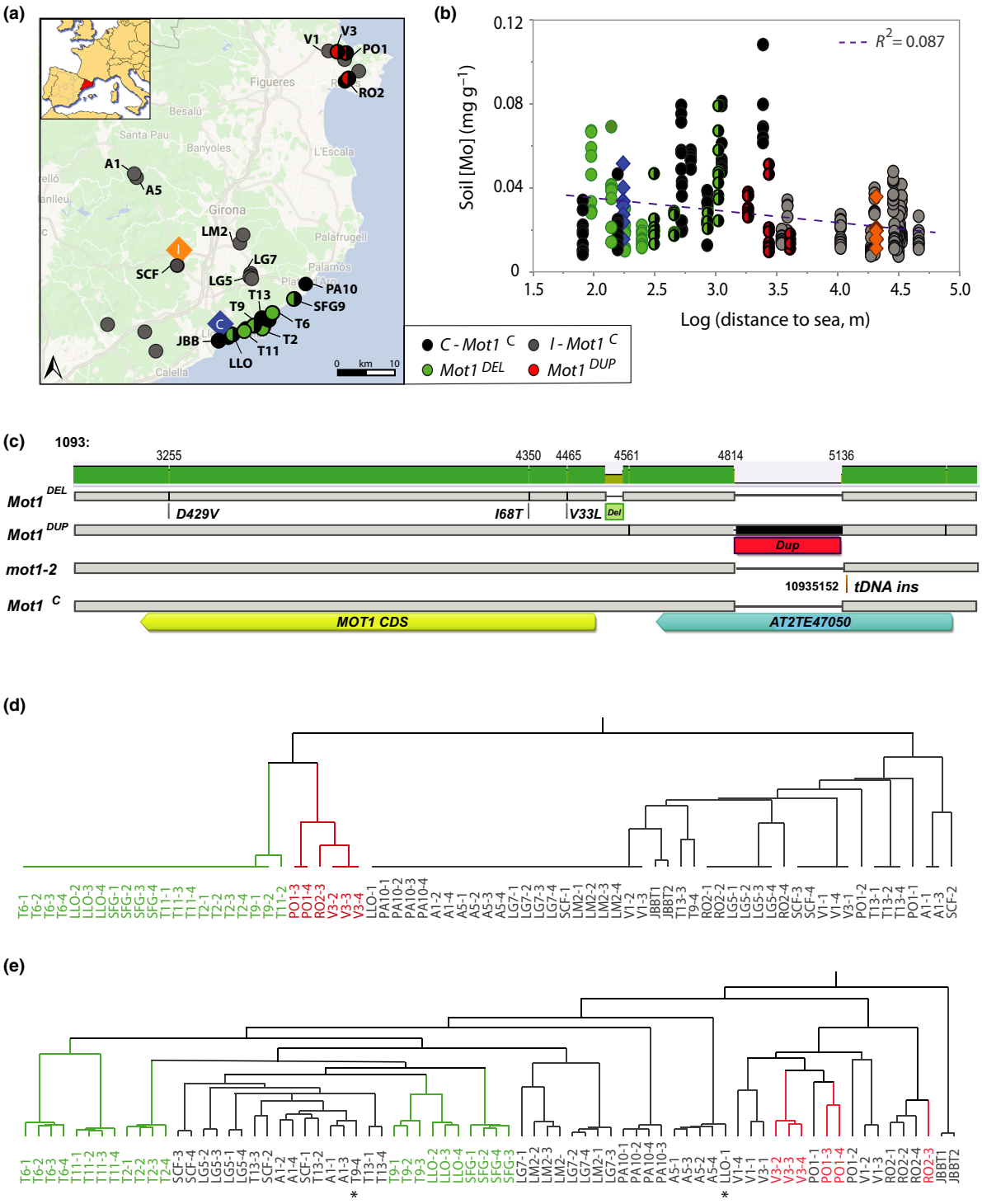

Fig. 1 Sampling, *MOT1* alleles studied, genetic structure, and relationship with soil molybdenum (Mo) concentrations. (a) Geographic location in Catalonia, north-east Spain of the 36 demes of Arabidopsis studied, with the 19 sequenced demes named. (b) Distribution of each *MOT1* allele as a function of distance to the sea and soil Mo concentration. The correlation between soil Mo and distance to the sea (dotted line) is not significant ($r^2 = 0.087$). Black dots, coastal demes consisting of $MOT1^C$; grey, inland demes consisting of $MOT1^C$; green, coastal demes consisting of $MOT1^{DEL}$; black/green, demes harbouring both $MOT1^C$ and $MOT1^{DEL}$; black/red, demes harbouring both $MOT1^C$ and $MOT1^{DUP}$. The rhombus symbols represent the two field sites, Blanes (coastal, blue rhombus) and Santa Coloma de Farners (inland, orange rhombus). (c) Sequence alignment of the *MOT1* locus for $MOT1^C$, $MOT1^{DEL}$, $MOT1^{DUP}$ alleles and *mot1-2* mutant (SALK_069683). Genomic positions, *MOT1* CDS, AT2TE47050 and polymorphisms are indicated. (d, e) Neighbour-joining cladograms of the *MOT1* allele (based on 262 SNPs) (d) and the whole-genome fourfold degenerate (putatively neutral) sites (37,574 SNPs) (e) among 74 Arabidopsis individuals from our study region in northeast Spain. In *MOT1*, the highest probability for the value of $K = 3$ classifies the three versions of the *MOT1* allele: $MOT1^C$ (black), $MOT1^{DEL}$ (green), $MOT1^{DUP}$ (red). The same colours have been used on the whole-genome cladogram, with asterisks (*) denoting individuals with the $MOT1^C$ allele from demes containing plants with the $MOT1^{DEL}$ allele.

plants in any inland deme, suggesting a possible cost of the allele under inland conditions.

We analysed the *MOT1* locus in 74 resequenced individuals from 19 demes chosen to represent the full range of ecotypic and genetic variation over this area (Dataset S1 in Busoms *et al.* (2018)). We also performed *MOT1* transcript expression analysis and quantified leaf Mo concentrations across selected demes (Fig. S1). In $MOT1^{DEL}$ individuals we observed a 53 bp deletion 13 bp upstream of the transcription start site; we also detected an additional nonsynonymous change in the coding region (Fig. 1c), and we confirmed the low leaf Mo content and the reduced expression of *MOT1* in both roots and leaves (Fig. S1). Individuals with the $MOT1^{DUP}$ allele have a 322 bp duplication inside of the transposable element AT2TE47050 (Fig. 1c) and a higher expression of *MOT1*, especially in roots, and the plants accumulate higher levels of Mo in their leaves (Fig. S1). Although Mo leaf content in $MOT1^{DEL}$ individuals is reduced, plants do not show symptoms of Mo deficiency: Mo concentrations are within the normal range (0.4–1.0 $\mu g\, g^{-1}$) for Brassicaceae species (Mengel *et al.*, 2001).

To gain an understanding of the population genomic relationships among plants harbouring each of the three major *MOT1* alleles, we generated a neighbour-joining (NJ) cladogram of the *MOT1* locus from our 74 resequenced individuals and compared it to the genome-wide cladogram. This group consisted of 22 individuals with $MOT1^{DEL}$; six with $MOT1^{DUP}$; and 46 with the $MOT1^{C}$ allele (Fig. 1d,e). As predicted, the *MOT1* locus cladogram differentiates the *MOT1* alleles into three distinct clades (Fig. 1d). Except for the LLO-1 individual that genetically clusters with the individuals from the A5 deme (suggesting a rare case of migration due to the demes being > 40 km apart, as previously discussed in Busoms *et al.* (2018)), Fig. 1(e) shows that genomes harbouring $MOT1^{DEL}$ or $MOT1^{DUP}$ are nested among $MOT1^{C}$ sisters, indicating that different *MOT1* alleles have recombined onto common genetic backgrounds.

MOT1 influence on coastal adaptation

Despite our dense sampling, we could only find the $MOT1^{DEL}$ allele in demes located < 3 km from the coast, suggesting that this allele may have a role in adaptation to coastal environments. To test this idea, we reanalysed data from reciprocal transplants performed in coastal and inland field sites in 2013 and 2014 (Busoms *et al.*, 2015), considering the status of the *MOT1* allele version in each individual plant. Fig. 2(a) shows that plants harbouring $MOT1^{DEL}$ are consistently better adapted to coastal conditions, often producing over twice as many siliques when grown at the coast and outperforming the rest of the coastal demes.

To isolate the effect of the *MOT1* allele and homogenise genetic backgrounds, we selected two mixed demes with plants harbouring $MOT1^{C\ or\ DEL}$ alleles ($T9^{C}$, $T9^{DEL}$; $LLO2^{C}$, $LLO2^{DEL}$) for further reciprocal transplant experiments at the same coastal and inland sites. In both years (2014 and 2015), plants with the $MOT1^{C}$ allele from both demes were less fit than plants with the $MOT1^{DEL}$ allele when grown at the coast. However, no difference was observed between plants with the two alternative alleles at the inland site (Fig. 2b), confirming coastal-specific adaptation of $MOT1^{DEL}$ harbouring plants. We have been unable to identify plants harbouring the $MOT1^{DEL}$ in our inland sampling, suggesting a potential fitness cost for this allele in inland environments. However, given our benign common garden field conditions, it is perhaps not surprising that we do not detect a fitness cost over a single generation. That said, we do see differences in fitness proxies between plants harbouring each *MOT1* allele as a function of salinity challenge (Fig. 3a,b), with a lack of sodium challenge associated with lower fitness in the $MOT1^{DEL}$ allele, supporting the idea of a fitness effect. These results underscore a strongly environmental context-dependence on fitness estimations of naturally evolved alleles.

During the 3 yr of the reciprocal transplants, we monitored the soil mineral nutrient and trace element content at each field site (Dataset S2). Inland and coastal soils consistently differed in Na^{+}, magnesium (Mg), phosphorus (P), zinc (Zn), Cu, Mo and rubidium (Rb) concentrations (Fig. 2c). However, the accumulation of these elements in plant tissues did not fully reflect the soil quantities. Instead, a difference emerged specifically related to Mo. At all sites $MOT1^{DUP}$ plants had high shoot Mo, while $MOT1^{DEL}$ plants had not only lower Mo, but also decreased Na^{+} (Fig. 2d). $MOT1^{DEL}$ plants were also able to uptake more Cu, P and K^{+} than $MOT1^{C}$ plants under coastal conditions (Fig. 2d,e). These results indicate that the allelic variation present in *MOT1* not only results in reduced Mo accumulation in the leaves, but is also linked to mechanisms that allow $MOT1^{DEL}$ plants to exclude Na^{+}. Furthermore, we can affirm that this low Mo concentration in $MOT1^{DEL}$ plants does not adversely affect their normal development, as these plants had better or equal growth and reproductive fitness compared to $MOT1^{C}$ plants when cultivated under natural field conditions, as measured by silique number (Fig. 2b).

MOT1 interaction with Na^{+} and Cu homeostasis

To confirm that the $MOT1^{DEL}$ allele can mediate a salinity resistance phenotype whilst maintaining low Mo and Na^{+} contents and high Cu content in leaves, we performed controlled environment experiments. We modulated NaCl concentrations in both soil and hydroponics using the same demes we had cultivated in the field ($T9^{C}$, $T9^{DEL}$; $LLO2^{C}$, $LLO2^{DEL}$). Without the addition of NaCl, plants containing $MOT1^{C}$ or $MOT1^{DEL}$ alleles grew similarly. After treatment with 50 mM or 100 mM NaCl for 3 wk, growth of all plants was reduced. However, plants with the $MOT1^{DEL}$ allele and the *mot1-2* mutant grew significantly better than plants with the $MOT1^{C}$ allele in high NaCl conditions (Fig. a). This enhanced salinity tolerance linked to low *MOT1* expression was also reflected in fitness. Both the *mot1-2* mutant and $MOT1^{DEL}$ plants cultivated in the same soil produced more siliques than Col-0 and $MOT1^{C}$ plants when they were irrigated with NaCl at any concentration (Fig. 3b). Ionomic data from these experiments confirmed that plants with the $MOT1^{DEL}$ allele subjected to salinity stress accumulate more Cu and less Na^{+} in their leaves than plants with the $MOT1^{C}$ allele under the same conditions (Fig. S2; Dataset S2).

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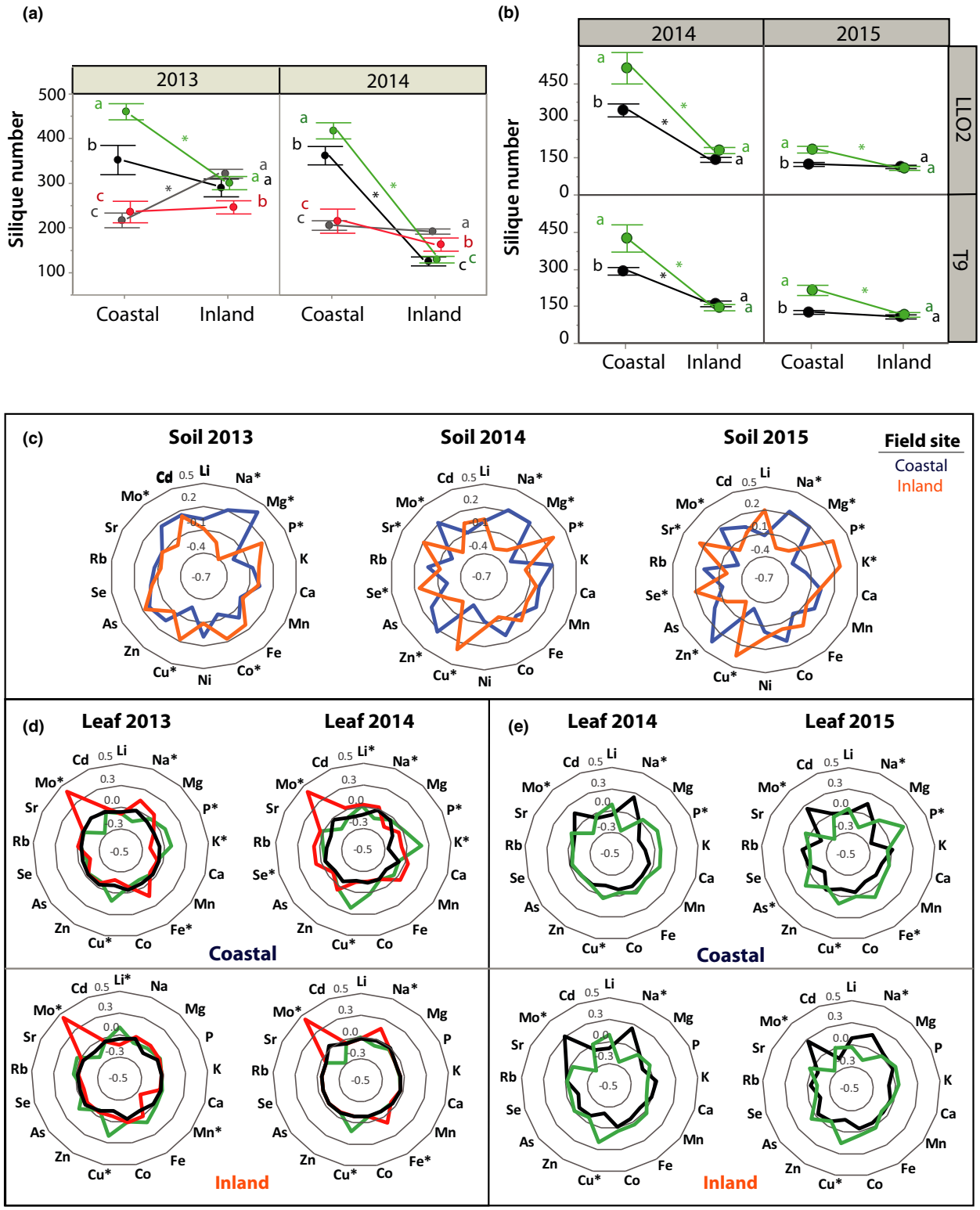

Fig. 2 Local adaptation of *MOT1* alleles and global elemental accumulation analysis. Fitness (mean ± SE of silique number) of (a) Arabidopsis plants from inland demes harbouring the $MOT1^{C}$ (grey) allele, and from coastal demes harbouring $MOT1^{C}$ (black), $MOT1^{DEL}$ (green) or $MOT1^{DUP}$ (red) alleles, cultivated at coastal and inland common gardens in 2013 ($n = 359$) and 2014 ($n = 400$); (b) plants from LLO2 and T9 demes harbouring the $MOT1^{C}$ (black) or $MOT1^{DEL}$ (green) alleles cultivated at coastal and inland common gardens in 2014 ($n = 40$) and 2015 ($n = 60$). Significant differences are indicated by letters (between alleles) and asterisks (between sites). Normalized difference of 17 elements in (c) soils from coastal (blue) and inland (orange) common gardens collected in the 3 yr of cultivations ($n = 60$); (d) leaves of plants harbouring the $MOT1^{C}$ (black), $MOT1^{DEL}$ (green) or $MOT1^{DUP}$ (red) alleles from 2013 ($n = 293$) and 2014 ($n = 390$) reciprocal transplants; and (e) leaves of plants from LLO2 and T9 demes harbouring the $MOT1^{C}$ (black) or the $MOT1^{DEL}$ (green) alleles from 2014 ($n = 40$) and 2015 ($n = 40$) reciprocal transplants. Elements exhibiting significant differences (according to a *t*-test) are marked with an asterisk (*, $P < 0.05$).

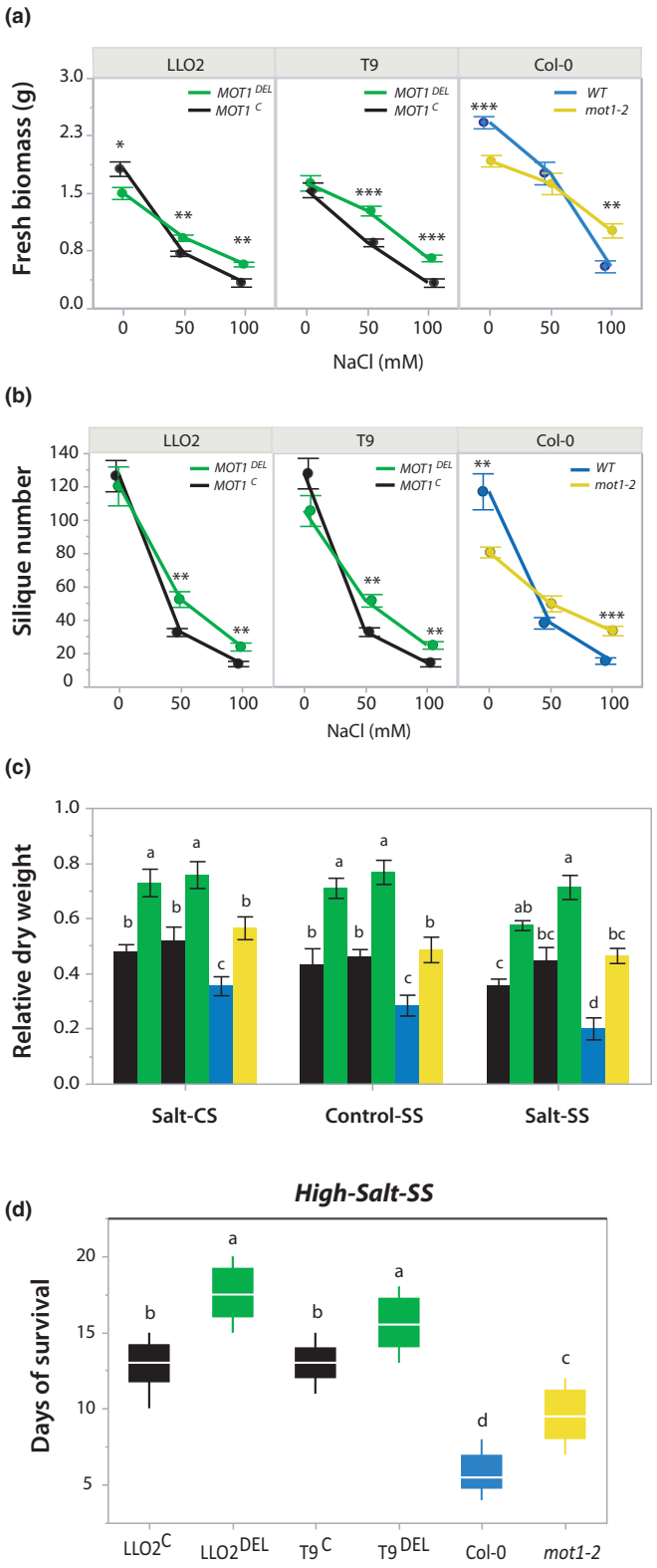

Fig. 3 Interplay of *MOT1* and salinity tolerance. (a) Fresh biomass (g) of Arabidopsis plants being exposed to either 0, 50 or 100 mM of NaCl in hydroponic solution for 3 wk and (b) fitness (number of siliques produced) of plants cultivated in potting mix soil under controlled conditions and irrigated with 0, 50 and 100 mM of NaCl until maturity. Data represent the mean ± SE of plants with the $MOT1^{C}$ (black) allele, plants with the $MOT1^{DEL}$ (green) allele from LLO2 and T9 demes, and *mot1-2* (yellow) and Col-0 (blue) individuals (10 plants per accession). Asterisks indicate significant differences (*t*-test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$). (c, d) Mean ± SE of (c) relative dry weight (DW treatment/DW control) and (d) survival (in days) after a high salinity treatment of plants with the $MOT1^{C}$ allele (black), plants with the $MOT1^{DEL}$ allele (green), and Col-0 (blue) and *mot1-2* (yellow) plants cultivated in potting mix soil irrigated with no NaCl (Control), 75 mM NaCl (Salt) or 150 mM NaCl (High-Salt) and sprayed with no NaCl (CS) or 150 mM NaCl (SS) for 2 wk. Bars represent the mean ± SE and boxplots represent the median and interquartile range of six plants per accession. Letters indicate significant differences (Tukey's HSD, $P < 0.05$).

We have previously established that coastal adaptation in the Arabidopsis demes we are studying here is driven primarily by adaptation to elevated soil salinity by assessing the fitness of plants grown in a controlled environment in excavated coastal and inland soil (Busoms *et al.*, 2015). However, in addition, we were interested in understanding whether the MOT^{DEL} can also confer elevated tolerance to salinity in the form of exposure to NaCl applied as a spray, to simulate salt spray from the sea (Du & Hesp, 2020). We therefore cultivated the same demes from the previous experiments in potting mix soil, and 3 wk after sowing we started five different treatments: Control-CS (plants irrigated and sprayed with 0 mM of NaCl), Salt-CS (plants irrigated with 75 mM and sprayed with 0 mM of NaCl), Control-SS (plants irrigated with 0 mM and sprayed with 150 mM of NaCl), Salt-SS (plants irrigated with 75 mM and sprayed with 150 mM of NaCl), and High-Salt-SS (plants irrigated with 150 mM and sprayed with 150 mM of NaCl). After 2 wk of treatment, plants from the first four treatments were harvested, dried and weighed. Plants from the fifth treatment were visually inspected to record survival. Compared to control plants, coastal demes with the $MOT1^{DEL}$ allele showed high tolerance to soil salinity, salt spray and the combination of both (Fig. 3c,d). Col-0 plants were highly sensitive to salt spray, dying a few days after the start of the High-Salt-SS treatment. By contrast, *mot1-2* plants grew less than $MOT1^{DEL}$ individuals but always performed better than Col-0 in all the salinity treatments, exhibiting a similar tolerance to plants with the $MOT1^{C}$ allele (Fig. 3c,d).

To functionally confirm the association between the *MOT1* allele and salinity in a broad diversity panel, we challenged a set of 306 natural accessions (14 with the $MOT1^{DEL}$ allele) with 50 mM NaCl 5 wk after sowing, and further added 25 mM NaCl each following wk for 2 wk. We measured leaf Na^{+} content before and after the treatment. This confirmed for a much broader sample that $MOT1^{DEL}$ plants accumulate less Na^{+} in aerial tissues when Na^{+} solution concentrations are high (Fig. 4a).

To further test whether the *MOT1* deletion is related to Na^{+} exclusion, we analysed the leaf ionome profiles of 953 accessions from the 1135 Arabidopsis collection (Campos *et al.*, 2021) after growth in nonsaline potting mix (Dataset S2). We classified them into three groups ($MOT1^{C}$: black; $MOT1^{DEL}$: green; $MOT1^{DUP}$: red) and located them on an online map (Fig. S3a). Geographical variables ('Country' and 'Distance to sea'), and 18 estimated soil parameters based on Land Use and Coverage Area frame Survey

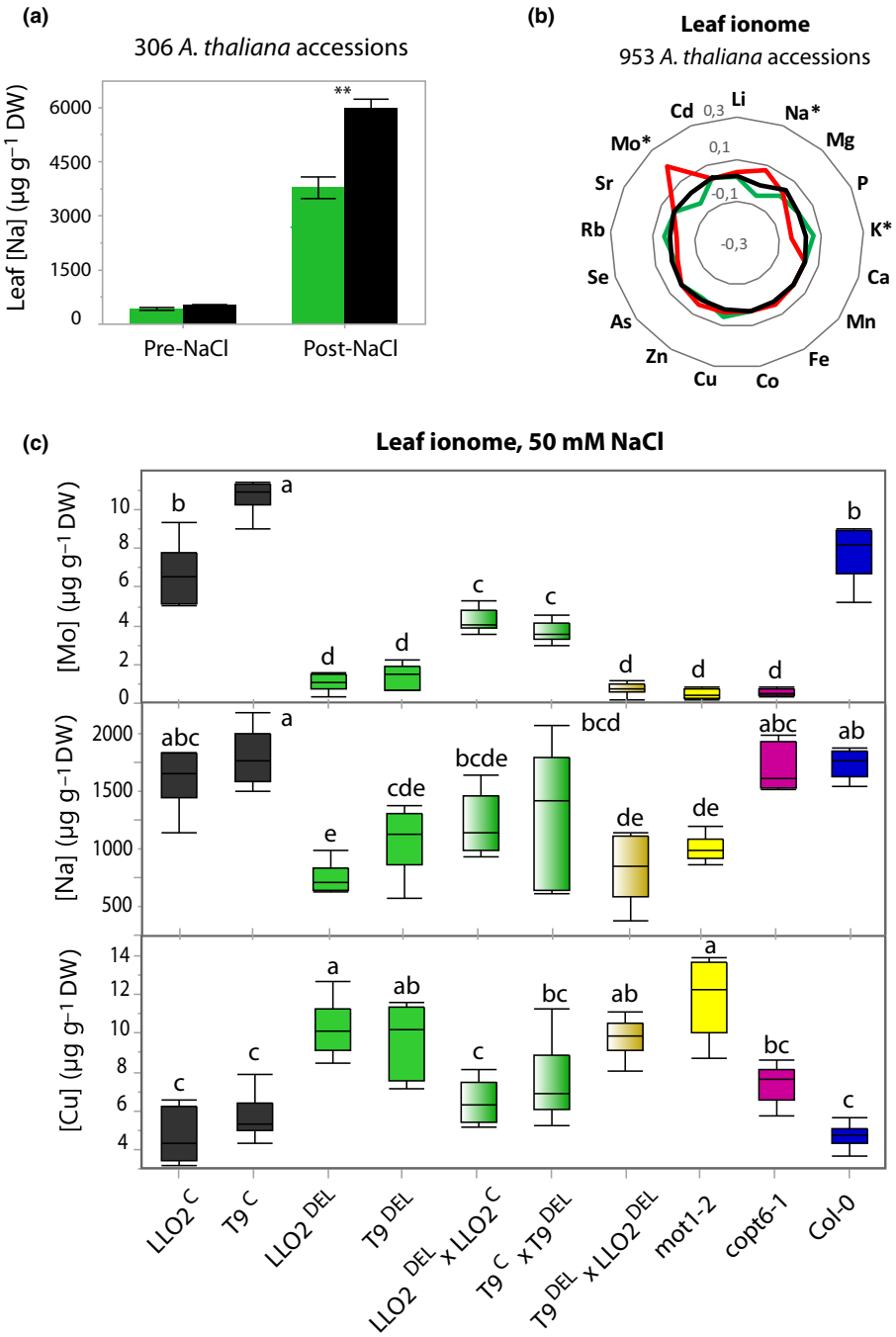

Fig. 4 Allelic effects on ion content. (a) Mean ± SE of sodium (Na^+) content in leaves of plants from 306 European Arabidopsis accessions (14 *MOT1*^{DEL} (green); 292 *MOT1*^{C} (black)) cultivated in control conditions for 5 wk, and leaves from the same plants after being irrigated with NaCl for 4 wk. Asterisks indicate significant differences between *MOT1* alleles (*t*-test; **, $P < 0.005$). (b) Normalized difference of 17 elements in leaves of 953 accessions from the Arabidopsis world-wide collection of 1135, classified into plants harbouring the *MOT1*^{C} (black), the *MOT1*^{DEL} (green) or the *MOT1*^{DUP} (red) alleles. (c) Mean ± SE of shoot molybdenum (Mo), Na^+ and copper (Cu) content (µg g^{-1} dry weight) of Col-0, T9^{C}, T9^{DEL}, LLO2^{C} and LLO2^{DEL} parental plants, F1 plants from crosses between T9^{C} × T9^{DEL}, LLO2^{DEL} × LLO2^{C} and T9^{DEL} × LLO2^{DEL} and *mot1-2* and *copt6-1* mutants cultivated in potting mix soil irrigated with 50 mM of NaCl for 4 wk. Boxplots represent the median and interquartile range of six plants per accession, and letters indicate significant differences (*F*-test, $P < 0.05$).

(LUCAS) 2009/2012 topsoil data (Panagos *et al.*, 2012) were tested. We could discern no clear association between the presence of *MOT1* allelic variation and these variables (Fig. S3b). However, leaf Mo and Na^+ content were significantly lower in plants with the *MOT1*^{DEL} allele when cultivated under common conditions (Fig. 4b), indicating a world-wide effect of the presence of these *MOT1* alleles on the accumulation of Mo and Na^+. We also observed elevated K^+ associated with *MOT1*^{DEL}.

The main interaction between Mo and Cu occurs via the synthesis of the Mo containing cofactor Moco, where COPT

transporters play a role. In particular, the Cu transporter *COPT6* has been associated with *MOT1* (Billard *et al.*, 2014; Forsberg *et al.*, 2015). To examine whether polymorphisms in *MOT1* and *COPT6* independently affect Mo and Cu concentrations *in planta,* we compared Mo and Cu concentrations in *mot1-2* and *copt6-1* plants cultivated in common soil irrigated with 50 mM NaCl for 3 wk (Fig. 4c). In both *mot1-2* and *copt6-1* mutants, leaf Mo contents are low in comparison to the wild-type Col-0. Moreover, the *mot1-2* mutant exhibited elevated leaf Cu levels, suggesting a potential link between these two genes (Fig. 4c).

To further confirm that the *MOT1* locus is responsible for the ionomic changes detected in the Catalonian demes from north-east Spain, we performed a complementation test, crossing $LLO2^{DEL} \times LLO2^{C}$, $T9^{C} \times T9^{DEL}$ and $T9^{DEL} \times LLO2^{DEL}$. F1 and parental plants were cultivated together with *mot1-2* and *copt6-1* mutants. F1 plants from the $LLO2^{DEL} \times LLO2^{C}$, $T9^{C} \times T9^{DEL}$ crosses contained intermediate concentrations of shoot Mo, Na^{+} and Cu relative to the parents (Dataset S2), indicating complementation. Only the F1s from the $T9^{DEL} \times LLO2^{DEL}$ cross had significantly lower shoot Mo and Na^{+} ($P < 0.005$) and higher Cu, similar to the $MOT1^{DEL}$ parents (Fig. 4c), confirming that $T9^{DEL}$ and $LLO2^{DEL}$ are allelic with the recessive loss of function *MOT1* allele.

MOT1 link to abscisic acid signalling and salinity tolerance

Soil ionome analyses showed that our coastal soils, as well as being rich in Na^{+} due to the marine influence (Busoms *et al.*, 2015), contain lower concentrations of Cu compared to inland soils (Fig. 5a). Such levels are not considered critical for normal plant growth but we detected that leaf Cu concentrations in the majority of plants with the $MOT1^{C}$ allele growing at the coast is low (between 1 and 7 $\mu g\, g^{-1}$), concentrations that may indicate Cu deficiency (Marschner, 1995). However, coastal plants with the $MOT1^{DEL}$ allele are able to accumulate more Cu inside the plant (Fig. 5b). Expression of *SPL7* and *COPT6* are elevated in plants containing $MOT1^{DEL}$, suggesting that these genes are playing a role in this elevated leaf Cu (Fig. 5c). This is also supported by the enhanced leaf Cu and *SPL7* expression we observed in the *mot1-2* mutant (Figs 4c, 5c). *SPL7* activates the transcription of multiple genes involved in Cu homeostasis in Arabidopsis (Yamasaki *et al.*, 2009; Jung *et al.*, 2012), and the *COPT6* plasma membrane transporter has previously been shown to be induced under Cu deficiency conditions (Garcia-Molina *et al.*, 2013; Puig, 2014; Peñarrubia *et al.*, 2015). As a consequence, $MOT1^{DEL}$ plants can maintain leaf Cu concentrations within an adequate range despite growing in soils with lower Cu concentrations, in contrast to $MOT1^{C}$ plants.

COPT6 is localized mainly in the vasculature of green tissues where it can facilitate Cu redistribution (Garcia-Molina *et al.*, 2013) and also plays an important role in ABA biosynthesis. Abscisic acid regulates *SPL7* and subsequently affects the expression of its *COPT* targets (Carrió-Seguí *et al.*, 2016). Noting the potential relationship between *COPT6* and *MOT1* (Forsberg *et al.*, 2015), we hypothesized that $MOT1^{DEL}$ may mediate a degree of salinity tolerance at the coast due to increased Cu concentrations and an enhanced ABA response. We therefore quantified the expression of the main genes involved in Moco biosynthesis, a critical cofactor in ABA biosynthesis, and ABA concentrations in plants with various *MOT1* alleles submitted to salt stress.

The formation of active Mo containing enzymes depends not only on the availability of Mo but also on the presence of the two metals iron (Fe) and Cu (Vigani *et al.*, 2017). It is assumed that Cu binds to the dithiolate group of molybdopterin (MPT) at the end of step 2 of Moco biosynthesis (Mendel, 2007). In this second step, MPT is initially formed by the incorporation of two sulfur atoms into cPMP in a reaction catalyzed by MPT synthase, a heterotetrameric *complex* of Cnx6 and Cnx7 subunits. The two small Cnx7 subunits are responsible for S transfer into the complex, but the MPT formation is mainly regulated by *CNX6* (Ide *et al.*, 2011). In the next step, MPT is adenylated by Mo insertase CNX1, forming MPT-AMP. In the last step, MPT-AMP is transferred to the Cnx1 E-domain, where Mo is inserted by replacing Cu, forming Moco, which is finally allocated to Mo apoenzymes (Kaufholdt *et al.*, 2013), such as aldehyde oxidase (AO). ABA3 is an enzyme required for the enzymatic activity of AO, the enzyme essential for the biosynthesis of bioactive ABA (Watanabe *et al.*, 2018). We observed that salt stress increased the expression of *CNX6*, *CNX1* and *ABA3* in all genotypes, but they were strongly upregulated in the *mot1-2* mutant and $MOT1^{DEL}$ plants (Fig. 5c). Consequently, ABA concentrations were also higher in $MOT1^{DEL}$ plants after salinity treatment (Fig. 5d), supporting the hypothesis that the $MOT1^{DEL}$ allele provides an enhanced ABA response to saline challenge.

We found that ABA concentrations in *copt6-1* mutants were significantly lower in all treatments (Fig. 5d), supporting the role of Cu in ABA biosynthesis. Under salinity stress, plants harbouring the $MOT1^{DEL}$ allele showed the highest induction of ABA biosynthesis (Fig. 5d). This may allow $MOT1^{DEL}$ plants to maintain their normal growth, even after being treated with 100 mM of NaCl (Fig. S4b).

At the cellular level, both Mo and Cu are required for Moco biosynthesis, but the amount of each element can act either positively or negatively during this process (Peñarrubia *et al.*, 2015). It has been reported that *CNX1* binds to the cytoskeleton (Schwarz *et al.*, 2000) and could have a role as a Mo or Cu sensor, interacting with *MOT1* or *COPT* transporters to regulate Mo and Cu concentrations in the cell (Tejada-Jimenez *et al.*, 2009). Sulfurylated Moco is a cofactor of ABA–AO that functions in the last step of ABA biosynthesis, and increased ABA concentrations being involved in responses to salinity has been well documented (e.g. Park *et al.*, 2016). Therefore, the crosstalk between Mo-Cu-Na^{+} via ABA synthesis may explain the greater tolerance to salinity of our plants harbouring the $MOT1^{DEL}$ allele (Fig. 6).

Conclusions

Complex interactions occur among the homeostatic controls of various nutrients in plants (Kearse *et al.*, 2012). However, a comprehensive understanding of the interactions between pathways integrating the homeostasis of key elements is still lacking,

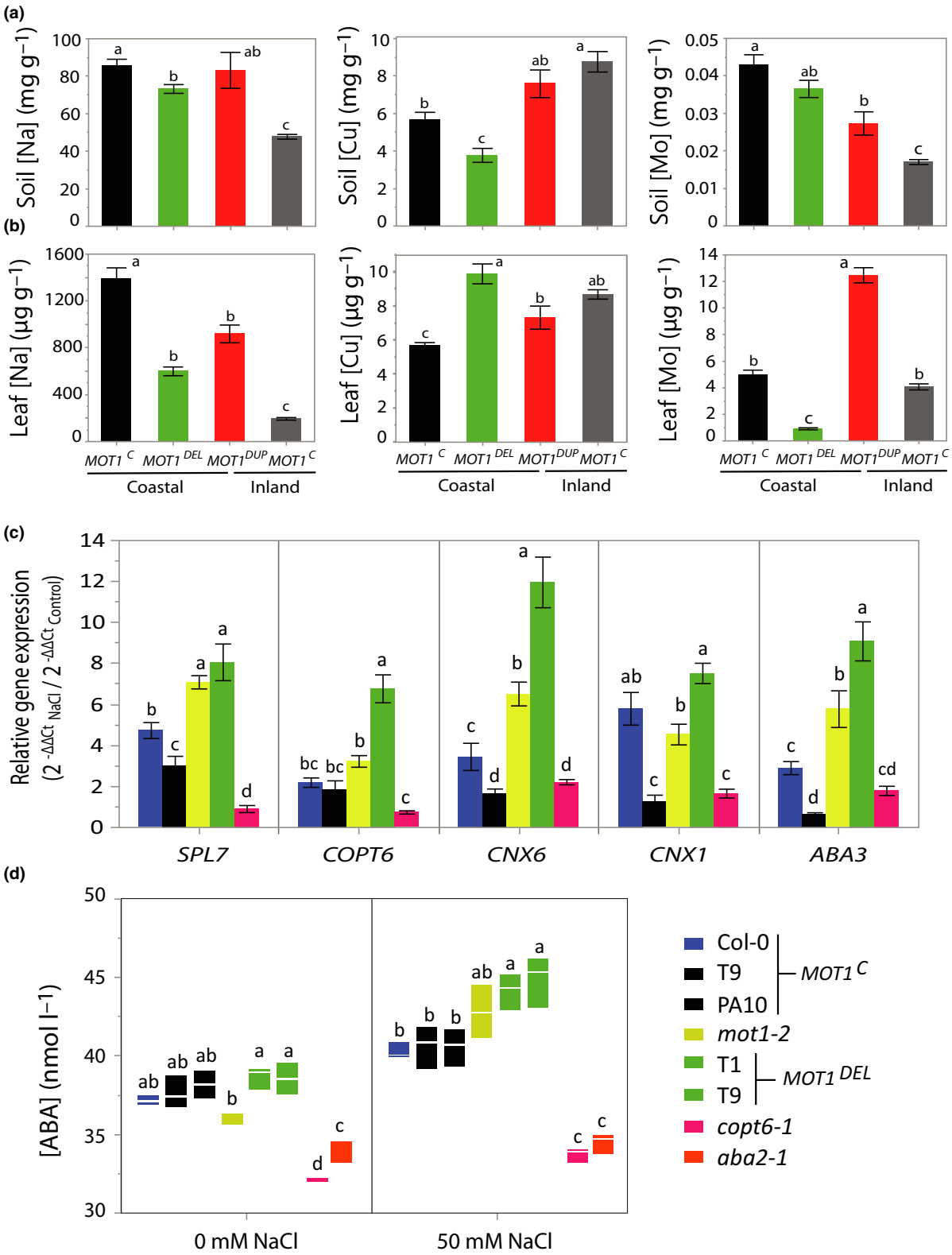

Fig. 5 Stress signalling and *MOT1*. (a) Mean ± SE of soil sodium (Na^+), copper (Cu) and molybdenum (Mo) content of nine soil samples per deme, and (b) mean ± SE of leaf Na^+, Cu and Mo content of Arabidopsis plants collected from their natural habitat, classified as coastal demes harbouring the $MOT1^C$ allele (black), coastal demes harbouring the $MOT1^{DEL}$ allele (green), demes harbouring the $MOT1^{DUP}$ allele (red) and inland demes harbouring the $MOT1^C$ allele (grey). (c) Relative transcript expression (treatment (50 mM NaCl) vs control (0 mM NaCl)) of *SPL7, COPT6, CNX6, CNX1* and *ABA3* in shoots of triplicate biological replicates of plants cultivated in hydroponics under control conditions or exposed to 50 mM of NaCl for 2 wk. Data represent the mean ± SE of three plants per accession. (d) Abscisic acid (ABA) content (nmol l^{-1}) of plants cultivated in common soil irrigated with 0 and 50 mM of NaCl for 2 wk. Boxplots represent the median and interquartile range of three plants per accession. Letters indicate significant differences (Tukey's HSD, $P < 0.05$).

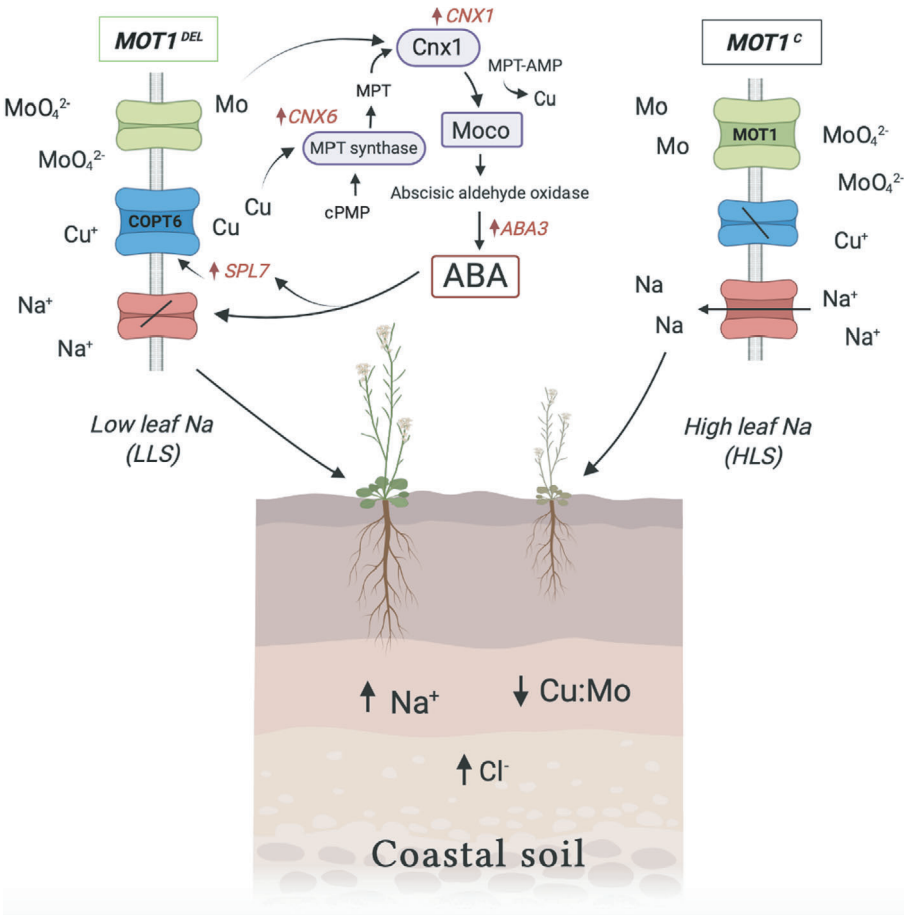

Fig. 6 Model for the salinity tolerance strategy of *MOT1*^{DEL} plants adapted to coastal conditions. ABA, abscisic acid.

especially as they apply to adaptive contexts in nature. Here we focused on allelic variation detected on the Arabidopsis *Molybdenum Transporter MOT1*, in particular on the most prevalent variant, *MOT1*^{DEL}, which is characterized by low *MOT1* expression and reduced leaf Mo content. We explored the adaptive value of this low expression in the context of the linked phenotype of altered Na^+ and Cu homeostasis. Within our study area in north-east Spain, Arabidopsis plants harbouring the *MOT1*^{DEL} allele could only be found near the salt-rich coast. Reciprocal transplant experiments demonstrated that plants with the *MOT1*^{DEL} allele are better adapted to these coastal conditions than those with the normal-functioning *MOT1*^{C} allele. Moreover, salt stress challenge experiments demonstrated that the *MOT1*^{DEL} allele is associated with enhanced salinity tolerance. Apart from Na^+, leaf Cu content was the only element that was consistently elevated under salinity challenge. This underscores the relationship between *COPT6* and *MOT1* and the importance of Cu in Moco and ABA biosynthesis. We accordingly suggest that *MOT1*^{DEL} has an adaptive advantage in coastal conditions, due to pleiotropic enhancement of both Cu and ABA levels.

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Author contributions

SB, CP and DES conceived the study. SB and JT performed field collections and laboratory experiments. SB and LY performed analyses. SB wrote the manuscript with primary input from all authors. All authors edited and approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 *MOT1* allele type classification and leaf molybdenum (Mo) content of the plants cultivated in the reciprocal transplants.

Dataset S2 Soil and leaf ionome analysis from all the experiments conducted in this study.

Dataset S3 *MOT1* allele type classification and coordinates of the world-wide set of 1135 Arabidopsis natural accessions.

Dataset S4 *MOT1* allele type classification and leaf sodium (Na^+) content of a world-wide set of 306 Arabidopsis natural accessions.

Dataset S5 Statistical analysis of all the comparisons performed in this study.

Fig. S1 Leaf Mo content and root and shoot *MOT1* expression.

Fig. S2 Ionomic profiles of *MOT1* alleles exposed to salinity

Fig. S3 Map and geostatistical analysis of the 1212 world-wide Arabidopsis accessions classified by *MOT1* allele type.

Fig. S4 Growth of plants with different *MOT1* alleles exposed to soil salinity and salt spray.

Methods S1 DNA and RNA extractions for *MOT1* genotyping and *target gene* expression.

Methods S2 Irrigation and hydroponic experiment procedures.

Methods S3 Salt spray and survival experiment design.

Methods S4 Salinity test design of 306 Arabidopsis world-wide accessions.

Table S1 Primers list for transcript quantification of target genes.

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